Isolation of the Human ATP-Creatine Transphosphorylases (Creatine Phosphokinases) from Tissues of Patients with Duchenne Muscular Dystrophy*

(Received for publication, January 14, 1977, and in revised form, June 9, 1977)

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Detailed procedures are described for the separation and isolation of the isoenzymes of ATP-creatine transphosphorylase (creatine phosphokinase) from autopsy samples of muscle and brain tissue of five terminal dystrophic males (x-linked, Duchenne). These direct isolation studies have led to the remarkable observation that three isoenzymes are present in the atrophied muscle tissue (characterized electrophoretically and immunologically as muscle type, hybrid type, and brain type, in order of decreasing amounts), but only one isoenzyme (brain type) is present in the dystrophic brain tissue. The muscle type was isolated in crystalline form, the other muscle isoenzymes (hybrid and brain type) were purified, the hybrid type was also purified from the heart tissue, and the brain type from the dystrophic brain was crystallized. In the normal male adult, however, only one isoenzyme appears to be present in the muscle (the muscle type) and only one isoenzyme (the brain type) in the brain. Also, studies on a stillborn and an 11-month-old male infant, revealed only the muscle type with traces of the hybrid type, and the absence of the brain type in the skeletal muscle. A sample obtained from the muscle of an 11- to 12-week-old female fetus also yielded the three-isoenzyme distribution pattern, characterized electrophoretically as muscle type, hybrid type, and brain type. Therefore, the isoenzymic distribution pattern of ATP-creatine transphosphorylase in the atrophied musculature of the terminal human progressive muscular dystrophic organism appears strikingly similar to that of the human fetal muscle.

ATP-creatine transphosphorylase (or creatine phosphokinase) has been of special interest to those engaged in research in the muscular dystrophies, since elevated enzymatic activities in the serum have proved to be of great assistance in the early diagnosis of these disorders (1-3) (see Tyler (4) for a review of the clinical applications) or in the detection of the female carrier in the sex-linked Duchenne type (referred to as x-linked) of progressive muscular dystrophy (5).

Since the ATP-creatine transphosphorylase enzyme had been isolated in crystalline form from this laboratory from the muscle and brain of several species, including man (Kuby et al. (6), Keutel et al. (7, 8)), it was a natural extension of these studies to attempt the isolation of this enzyme from human dystrophic tissues, with the eventual goal of determining whether a structural alteration of this enzyme or enzymes had taken place which might be characteristic of this disease.

This report will deal specifically with the separation and isolation of the several isoenzymes of ATP-creatine transphosphorylase from the atrophied muscle and from the brain tissue of five terminal patients with x-linked progressive muscular dystrophy (Duchenne). Only a preliminary characterization will be presented here of the several isoenzymes, i.e. by their electrophoretic behavior and by a preliminary immunological study. In a succeeding report, a physicochemical and kinetic comparison will be drawn between the dystrophic isoenzymes and their normal human counterparts.

Preliminary reports have been presented (Jacobs et al. (9), Palmieri et al. (10)).

EXPERIMENTAL PROCEDURES

Materials

Autopsy material from five terminal dystrophic male patients, diagnosed (e.g., Ref. 11) as progressive muscular dystrophy (x-linked, Duchenne), was the source of the tissues for the isolation studies described below. The weighed tissues (in plastic bags) were frozen in liquid nitrogen immediately following the autopsy and stored at −15° until ready for isolation. All other materials, including the substrates, Sephadex G-100 and G-200, purification reagents (e.g. (NH₄)₂SO₄, 99% ethanol, β-mercaptoethanol, the acid-base cycling of DEAE-cellulose (Bio-Rad Cellulose-D, exchange capacity 0.66 meq/g), phosphocellose (Bio-Rad Cellulose-P, exchange capacity 0.88 to 1.00 meq/g), DEAE-Sephadex A-50 (Pharmacia, exchange capacity ~3.5 meq/g), and their storage) were as described for the isolation of the calf brain enzyme (7).

* This work was supported in part by grants from the National Science Foundation, The National Institutes of Health, and the Muscular Dystrophy Association. This is the first paper in a series on "Studies on Muscular Dystrophy." The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 S. A. Kuby, R. H. Palmieri, K. Okabe, R. H. Yue, A. Frischat, and M. C. Cress, manuscript in preparation.
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Methods

Measurements of Enzymatic Activity—To follow the purification of the several isoenzymes, the original colorimetric procedure for creatine phosphate and reaction mixture conditions, as well as the definition of activity (1 mm unit of an antiserum type, pH 8.0), were employed (6) with a slight modification in the dilution of the enzyme (see Ref. 7). In addition, the pH-stat method (12, 13) with the reaction mixture for the forward reaction (7, 13–15) and the spectrophotometric (coupled enzyme assay, Ref. 16) method for the reverse reaction at 30°C (7, 14, 15), as described by Okabe et al. (17) were used. Concentrations in a 3.0 ml reaction mixture for the latter method (6) were: 2.1 x 10^-3 M ADP, 4.3 x 10^-3 M Mg(Ac), 3.3 x 10^-3 M creatine phosphate, 6.7 x 10^-3 M glucose, 6.7 x 10^-3 M NADP, pH 7.4 at 30°, 2 units of glucose-6-phosphate dehydrogenase and of hexokinase. For these latter two methods, 1 enzyme unit was defined as 1 μg/min, or 1 μmol/min, respectively. The ratio of units determined under colorimetric conditions, to pH-stat defined unit, to that obtained spectrophotometrically (coupled enzyme assay) was approximately 1:2.5:5 for practically all of the brain and muscle isoenzymes.

Protein was determined by a biuret procedure (18) or in later stages by its absorbance at 280 nm. Approximate biuret factors and extinction coefficients for some fractions of the normal human isoenzymes were given in Kauatal et al. (6) and assumed to apply here. Values for the isolated isoenzymes will be presented later.

Isoenzyme Distributions—Isoenzyme distributions were monitored by electrophoresis (19) on cellulose acetate (Serophore III, 2.5 μM), glucose (3.7 μM), MgSO₄ (4.1 μM), NADP (1.2 μM), hexokinase, phenazine methosulfate (0.2 mg/ml), nitrotetrazolium blue (0.2 mg/ml), and Tris/HCl (0.5 μM) relative amounts of each isoenzyme were estimated after elution of these latter two methods, 1 enzyme unit was defined as 1 μg/min, or 1 μmol/min, respectively. The ratio of units determined under colorimetric conditions, to pH-stat defined unit, to that obtained spectrophotometrically (coupled enzyme assay) was approximately 1:2.5:5 for practically all of the brain and muscle isoenzymes.

Antigen—Rabbit antisera against normal human muscle and brain type creatine kinases were obtained, after a long period of hyperimmunization, by a multiple-site intradermal injection technique similar to that described by Arnon and Shapira (20). Details are given in Ref. 21. The rabbit was initially injected with several milligrams of the respective crystalline normal human enzyme (isolated as described in Ref. 8) in 0.2 ml TFS solution (0.05 M Tris, 0.075 M KCl, 0.025 M HCl, 0.1 mM EDTA, 1 mM β- mercaptoethanol, pH 8.5) at 150 V (7 to 10 mA) for 60 min. Enzymatically active components were detected by application of a coupled enzyme dye mixture (8), via an overlying strip procedure (19). The relative amounts of each isoenzyme were estimated after elution of their respective areas, which were identified from parallel-run stained strips. Intact cutout area was then eluted by immersion in 1 ml of 50 mM Tris (Cl), 1 mM dithiothreitol, 1 mM EDTA, pH 7.0, at 0°C for several hours, with occasional agitation, followed by enzymatic assay of an aliquot by the spectrophotometric (coupled enzyme assay) method. Percentage recoveries were calculated using control strips.

Immunological Procedures—Rabbit antisera against normal human muscle and brain type creatine kinases were obtained, after a long period of hyperimmunization, by a multiple-site intradermal injection technique similar to that described by Arnon and Shapira (20). Details are given in Ref. 21. The rabbit was initially injected with several milligrams of the respective crystalline normal human enzyme (isolated as described in Ref. 8) in 0.2 ml TFS solution (0.05 M Tris, 0.075 M KCl, 0.025 M HCl, 0.1 mM EDTA, 1 mM β-mercaptoethanol, pH 8.5) at 150 V (7 to 10 mA) for 60 min. Enzymatically active components were detected by application of a coupled enzyme dye mixture (8), via an overlying strip procedure (19). The relative amounts of each isoenzyme were estimated after elution of their respective areas, which were identified from parallel-run stained strips. Intact cutout area was then eluted by immersion in 1 ml of 50 mM Tris (Cl), 1 mM dithiothreitol, 1 mM EDTA, pH 7.0, at 0°C for several hours, with occasional agitation, followed by enzymatic assay of an aliquot by the spectrophotometric (coupled enzyme assay) method. Percentage recoveries were calculated using control strips.

 RESULTS

To facilitate the description of the isolation of these several enzymes, the isolation procedures are divided into three sections: isolation of the isoenzymes from muscle tissue, from heart tissue, and from brain tissue. In the third section, the isolation procedure basically follows that already described for the calf brain enzyme (7), with the minor modifications indicated for the normal human brain tissue (8). Thus, only a flow sheet of the preparation is presented, together with only those few additional modifications which have been introduced in the preparation of the dystrophic brain enzyme.

All steps were carried out in a cold room (3-4°C) or in an ice bath unless otherwise specified (e.g. −10°C for the ethanol steps); pH measurements were made at or near 5°C with a Radiometer TTT1a meter, equipped with scale expander PHA 630 Ta, and A. H. Thomas glass calomel electrodes. The required amounts of solvent and (NH₄)₂SO₄, as well as the concentrations of these reagents were estimated by the formulas given, e.g. Ref. 26 (cf. 27–29). The technique of continuous flow dialysis is described in Ref. 27. A saturated solution of (NH₄)₂SO₄ was taken to be 3.9 M at 0°C.

Isolation of Muscle Type, Brain Type, and Hybrid Type from Dystrophic Skeletal Muscle Tissue—During the first trial, several unusual properties of the atrophied dystrophic skeletal muscle became immediately apparent. The procedure which had been developed for the isolation of the normal human muscle ATP-creatine transphosphorylase (see Ref. 8), especially the first two steps, and which had been modeled after the original isolation procedure of the rabbit muscle enzyme (6, 29) required alteration. The first sample of "muscle" tissue (−124 g) had been secured by autopsy immediately following death and frozen in liquid nitrogen; 4 days elapsed prior to the preparation. Attempts to homogenize the sample with 2 ml/g of 0.01 M KCl as before (29) yielded much too thick a suspension; the tissue was fibrous, dehydrated and especially blanched in color, indicative of very little myoglobin. A sample finally homogenized with 5 ml/g resulted in a manageable suspension, which after centrifugation yielded a lightly reddish extract with an unusually high pH of ~8.3 (measured at 5°C), whereas normal human muscle extracts have pH values of ~6 (see Ref. 8). This higher pH may also be indicative of a relatively slow glycolytic system with little lactic acid production. Lastly, electrophoresis on cellulose acetate (8) revealed a remarkable fact that there were three isoenzymes present in the extract in contrast to the one (the
A. Normal Male (0-16 Years)
B. Normal Male (0-11 Months)
C. Still-Born (0-9 Months)
D. Dystrophic (See-Linked Duchenne) Male (0-13 Years)
E. Isolated Muscle Isoenzyme
F. Isolated Hybrid Isoenzyme
G. Isolated Brain Isoenzyme

Fig. 1. A, cellulose acetate electrophoresis of human isoenzymes from normal and dystrophic muscle (see text for explanation). Slide D represents the electrophoretic pattern obtained from Fraction III of Preparation II and the three isoenzymes are identified, by comparison with the isolated normal human counterparts (Slides E, F, and G) as muscle type, hybrid type, and brain type in the ratio of 60:30:10. The enzymatically active isoenzymes have been stained for activity (see Ref. 8). B, cellulose acetate electrophoresis of human progressive muscular dystrophic isoenzymes from the muscle and brain tissues (see text for explanation). C, electrophoretic pattern of the isoenzymes of ATP-creatine transphosphorylase derived from the muscle of an 11- to 12-week-old fetus (female). The three active isoenzymes are identified as muscle type (M-M), hybrid type (M-B), and brain type (B-B); 5'-AMP (1 mM) had been included in the stain to inhibit the enzymatic activity of myokinase which underlies the muscle type band. The sex of the fetus was identified cytogenetically.
which shows remarkable similarity to its crystalline normal human muscle type counterpart) was essentially the same as that of the best preparations of crystalline normal muscle type, reported earlier (8). It was likely obtained in better than 80% overall yield from the initial starting material, but there is some inherent uncertainty in the estimation of the percentage of M-M' in the initial homogenate. In addition, dystrophic tissue (Table I (S)) on a wet weight basis contains only one-tenth the amount of total enzyme activity found in normal muscle (see Table I, Ref. 8), increasing the difficulties in developing an isolation procedure.

The M-B Fractions VII-a (which proved to be relatively impure) were pooled from Preparations II, III and IV, and the attempts to purify it further are summarized in Table II (S) of the Supplement. It finally was induced to crystallize, but further recrystallizations to constant specific activity were not attempted since the amount of material was now quite limited. Electrophoresis on cellulose acetate yielded only a single enzymatically active band of the hybrid type (see Fig. 1B (P), Slide D).

Isolation of Hybrid Type and Muscle Type from Dystrophic Heart Tissue—The details of the preparation from 100 g of heart tissue derived from a 19-year-old dystrophic male are provided in the supplement, where, by Fraction III, a total of approximately 9400 units were recovered and found to be distributed into ~85% muscle type, ~12% hybrid type, and ~3% brain type. The three isoenzymes were separated by DEAE-cellulose chromatography, at pH 7.0, with a Cl- gradient, as described in Fig. 6 (S). A trace of another enzymatically active band is observed which is situated between the hybrid type and brain type and which might be the "mitochondrial creatine kinase" (see Ref. 31). The conditions of the initial extraction at low ionic strength, viz. 0.01 M KCl, might not yield any more than traces of this enzyme. The small amount of hybrid type, recovered in good yield from the heart, appeared reasonably pure, but it was not obtained in sufficient amounts to attempt its crystallization. It was recovered in sufficient amounts, however, for antienzyme titrations, see Fig. 2C (P), where it can be seen that both the anti-M-M and the anti-B-B yield inhibition endpoints are curiously greater than 50% for each antienzyme (also, cf. Ref. 32). Inhibition titrations of the purified dystrophic muscle type (from the muscle) and brain type (from the brain) will also be described below (Fig. 2 A and B (P)), where it is shown that the dystrophic isoenzymes, like their normal human counterparts, are inhibited almost 100% by their respective antienzymes.

Isolation of Brain Type from Dystrophic Brain Tissue—Since only a single isoenzyme could be detected in the dystrophic brain (the brain type), the isolation procedure followed, with only slight changes (as described in the supplement), that which was described earlier for the calf brain (7) and for the normal human brain (8), and which are summarized by means of a flow sheet (see Scheme II (S)) and "Table of Recoveries and Purification" (Table III (S)). Also, since the objective was to isolate sufficient quantities of the dystrophic brain type for further chemical and physical characterization and comparison with the normal human counterpart, the brain tissue (~4800 g) was pooled from four terminal male dystrophies (ages 16, 13/4, 11, and 17 years) which had been stored in the frozen state (~20°) from 4 to 12 months. The details for the isolation procedure are given in the supplement.

4 The abbreviations used are: M-M, muscle type; B-B, brain type; M-B, hybrid type.

but it is pertinent here to mention that Fraction II (see Scheme II (S)), by cellulose acetate electrophoresis (see Slide E of Fig. 1B (P)), revealed the presence of only one enzymatically active component (plus adenylyl kinase), which was identical in electrophoretic mobility with the normal crystalline brain type from brain tissue (cf. Slide G of Fig. 1A (P)). The enzyme following Fraction VI (see Scheme II (S)) readily crystallized by dialysis (under nitrogen) against 47% saturated (NH4)2SO4, 10 mM EDTA, 10 mM β-mercaptoethanol and stored in liquid N2 for future studies. A summary of the preparation is provided in Table II (S) which demonstrates an almost quantitative recovery after well over a 100-fold purification, with a final specific activity of ~320 units/mg (pH-stat assay) or certainly one of the highest specific activities for a
human brain creatine kinase preparation reported (cf. Table IV of Ref. 8). In Fig. 1B (P) Slide F, only a single enzymatically active band is observed for the final preparation by cellulose acetate electrophoresis, which corresponded in mobility to the crystalline human brain type (cf. Fig. 1A (P)). Also a comparison with the normal human brain type (Table IV of Ref. 8) reveals that the yield of enzymatic activity of the brain type ATP-creatine transphosphorylase from the dystrophic brain was comparable to that obtained from the normal human brain.

Immunological Behavior and Comparison of Isolated Dystrophic Isoenzymes with their Respective Normal Counterparts — The partially purified antibodies against the normal human muscle and brain type creatine kinases act as both precipitating antibodies and as inhibiting antienzymes (cf. Fig. 2 (P) above). Thus, in Fig. 3, A and B (P), by immunoelectrophoresis, or by immunodiffusion (3C (P)), these two antibodies seem to be specific for either the brain type or the muscle type polypeptide chain of the human ATP-creatine transphosphorylase; and both antibodies react with the respective polypeptide chains of the two-chain hybrid type (Fig. 3A (P); also, cf. Ref. 33). In fact, although there is better than a 70% homology between the covalent structures of the muscle type creatine kinases of man and calf,5 the purified antihuman muscle type enzyme shows an unusual degree of specificity towards the human muscle type of polypeptide chain, both as an antienzyme (Fig. 2A (P)) and as a precipitating antibody (Fig. 3C (P)). By these precipitin tests, the dystrophic muscle type (from the muscle) seems to show a close identity to its normal human counterpart (see especially Fig. 3C (P), immunodiffusion studies). The dystrophic hybrid type (from the muscle) by these immunological tests can be considered to contain one polypeptide chain of muscle type and one of brain type (Fig. 3A (P) and Fig. 2C (P)) which appear to be similar to their normal counterparts. A comparison of the antihuman muscle enzyme reactions with normal human and dystrophic human muscle type creatine kinase (Fig. 2A (P)) also shows surprising similarity in inhibition titrations. As may be seen in Fig. 2A (P), there seems to be essentially no reaction of the antihuman muscle globulin with the normal human brain type, and the calf muscle creatine kinase requires more than a 100-fold excess of antibody to yield the same percentage inhibition as with the normal human muscle type enzyme, again indicative of an unusual type of specificity in these antibody reactions. In Fig. 2B (P), inhibition titrations of the normal and dystrophic brain types (from the brain) again yield similar titration curves, reflecting a similarity in structure.

Thus, although a comparison by electrophoresis on cellulose acetate (Fig. 1, A and B (P)) and in immunological tests (Fig. 2, A, B and C (P) and Fig. 3, A, B, and C (P)) of the isolated individual normal human isoenzyme counterparts, with their respective dystrophic counterparts reveals a great deal of similarity, the final proof of identity or nonidentity must await further definitive studies by physical, chemical, and kinetic means. Any covalent alterations in their structures

5 R. H. Palmieri and S. A. Kuby, unpublished observations.

Fig. 3. Immunoelectrophoresis and immunodiffusion of normal and dystrophic human creatine kinases from muscle and brain (at pH 8.5, 0.05 ionic strength). A, immunoelectrophoresis on agarose gel of normal and dystrophic human creatine kinases from muscle and brain. B, immunoelectrophoretic patterns on agarose gel of human creatine kinases against purified rabbit antihuman muscle type creatine kinase globulin. C, immunodiffusion through agarose gel of several ATP-creatine transphosphorylases against purified rabbit antihuman muscle type creatine kinase globulin.
which might reflect a mutational event must await the final elucidation of their total amino acid sequences.

It is clear, therefore, that by direct isolation studies and by their characterization electrophoretically and immunologically, that surprisingly, three isoenzymes, a muscle type, a hybrid type, and a brain type, were found in the atrophied skeletal muscle of terminal patients with x-linked progressive muscular dystrophy. But only one isoenzyme may be found in the brain of the terminal dystrophic human male, viz. the brain type. In the dystrophic heart tissue, in addition to the muscle type, both hybrid type and brain type are present. In contrast to the dystrophic organism, normal male human adults of the same age show only one isoenzyme in the skeletal muscle, the muscle type and only one within the brain, the brain type (but with muscle and hybrid type in the heart). It is most striking and pertinent to draw a comparison of the dystrophic muscle's three-isoenzyme distribution with the isoenzyme pattern observed in the fetal state (Fig. 1C (P)). One can only speculate at this point that the x-linked Duchenne progressive muscular dystrophy may be an inborn error in the biochemical mechanism of the muscular tissue in the human male organism, or, in essence, a disturbance in the biochemical regulatory mechanisms which govern the transition from fetal to adult behavior (cf. Refs. 34–37, on the ontogeny of the creatine kinase isoenzymes).

Acknowledgments—The assistance of the following individuals in the preparations is gratefully acknowledged: Ms. Carol Perry, Mr. Stephen Hofmeister, Ms. Carol Perry, Mr. Stephen Hofmeister, Mr. David Maugham, Mr. L. Maland, Mr. Gerald Fleming, Dr. W. Zundel for his assistance in securing the autopsy samples is acknowledged; and we are indebted to Dr. W. C. Wisor for his cyrogenetic analysis of the 11- to 12-week-old fetus. We are grateful to the Pathology Department of the University of Utah, College of Medicine, especially Drs. Cathey and Bloom, for the assistance with the autopsy material.

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Fig. 3. Chromatography of Reaction 19 of 20. The main peaks were eluted at 21.1 min. A. A peak of 20.1 min. was eluted at 24.1 min. B. A peak of 20.1 min. was eluted at 23.1 min. C. A peak of 20.1 min. was eluted at 22.1 min. D. A peak of 20.1 min. was eluted at 21.1 min. E. A peak of 20.1 min. was eluted at 20.1 min. F. A peak of 20.1 min. was eluted at 19.1 min. G. A peak of 20.1 min. was eluted at 18.1 min. H. A peak of 20.1 min. was eluted at 17.1 min. I. A peak of 20.1 min. was eluted at 16.1 min. J. A peak of 20.1 min. was eluted at 15.1 min. K. A peak of 20.1 min. was eluted at 14.1 min. L. A peak of 20.1 min. was eluted at 13.1 min. M. A peak of 20.1 min. was eluted at 12.1 min. N. A peak of 20.1 min. was eluted at 11.1 min. O. A peak of 20.1 min. was eluted at 10.1 min. P. A peak of 20.1 min. was eluted at 9.1 min. Q. A peak of 20.1 min. was eluted at 8.1 min. R. A peak of 20.1 min. was eluted at 7.1 min. S. A peak of 20.1 min. was eluted at 6.1 min. T. A peak of 20.1 min. was eluted at 5.1 min. U. A peak of 20.1 min. was eluted at 4.1 min. V. A peak of 20.1 min. was eluted at 3.1 min. W. A peak of 20.1 min. was eluted at 2.1 min. X. A peak of 20.1 min. was eluted at 1.1 min. Y. A peak of 20.1 min. was eluted at 0.1 min. Z. A peak of 20.1 min. was eluted at -0.1 min.

Fig. 4. Chromatography of Reaction 19 of 20. The main peaks were eluted at 21.1 min. A. A peak of 20.1 min. was eluted at 24.1 min. B. A peak of 20.1 min. was eluted at 23.1 min. C. A peak of 20.1 min. was eluted at 22.1 min. D. A peak of 20.1 min. was eluted at 21.1 min. E. A peak of 20.1 min. was eluted at 20.1 min. F. A peak of 20.1 min. was eluted at 19.1 min. G. A peak of 20.1 min. was eluted at 18.1 min. H. A peak of 20.1 min. was eluted at 17.1 min. I. A peak of 20.1 min. was eluted at 16.1 min. J. A peak of 20.1 min. was eluted at 15.1 min. K. A peak of 20.1 min. was eluted at 14.1 min. L. A peak of 20.1 min. was eluted at 13.1 min. M. A peak of 20.1 min. was eluted at 12.1 min. N. A peak of 20.1 min. was eluted at 11.1 min. O. A peak of 20.1 min. was eluted at 10.1 min. P. A peak of 20.1 min. was eluted at 9.1 min. Q. A peak of 20.1 min. was eluted at 8.1 min. R. A peak of 20.1 min. was eluted at 7.1 min. S. A peak of 20.1 min. was eluted at 6.1 min. T. A peak of 20.1 min. was eluted at 5.1 min. U. A peak of 20.1 min. was eluted at 4.1 min. V. A peak of 20.1 min. was eluted at 3.1 min. W. A peak of 20.1 min. was eluted at 2.1 min. X. A peak of 20.1 min. was eluted at 1.1 min. Y. A peak of 20.1 min. was eluted at 0.1 min. Z. A peak of 20.1 min. was eluted at -0.1 min.
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