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TOWARDS A DEFINITIVE ASSAY OF CREATININE IN SERUM AND IN URINE: SEPARATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A fast and sensitive method for the separation of serum and urinary creatinine is described. For the preliminary purification of serum and urine, a cation-exchange column is used to remove protein, anions and neutral compounds prior to isolation of creatinine by high-performance liquid chromatography. A reversed-phase system with 0.01 *M* ammonium acetate solution as the mobile phase can separate creatinine in 7.5 min at a flow-rate of 1 ml/min. The purity of the separated creatinine is proved by derivatization using trifluoroacetic anhydride, followed by gas chromatography and mass spectrometry.

Although this method of purification was designed for incorporation into a definitive assay, the ease and speed of analysis makes it very attractive for routine clinical use.

INTRODUCTION

The concentrations of creatinine in serum and in urine are commonly estimated in clinical laboratories since their ratio for a given patient affords a quantitative index of renal function. The majority of assay methods are based on the Jaffe reaction [1] in which creatinine reacts with alkaline picrate to form an amber-yellow colour. This technique, however, is non-specific, and many compounds are known to react with picric acid or cause interference [2]. Preliminary purification of creatinine by ion-exchange chromatography has been used in efforts to overcome these difficulties [3—8], but it is unlikely that such a method will abolish all interferences.

We are interested in developing a definitive method of assay [9] with high specificity and accuracy, based on stable isotope dilution—mass spectrometry in a similar way to that described for serum calcium [10] and serum phosphate [11]. The development of such a method as for uric acid [12] would necessitate the isolation of pure creatinine and the preparation of a volatile

derivative for gas chromatography and mass spectrometry. Tris(trimethylsilyl)creatinine has been prepared [13] by treating creatinine with N-methyl-N-trimethylsilyltrifluoroacetamide and chromatographed on a 3% OV-1 column. This derivative, however, was found to be extremely sensitive to moisture and was unsuitable for accurate isotope-dilution work as its mass spectrum had too many interfering peaks due to the many naturally occurring isotopes of silicon. A new derivative was therefore required.

In this paper we describe the separation of creatinine from biological fluids by high-performance liquid chromatography (HPLC) and the synthesis of O-trifluoroacetylcreatinine. The HPLC separation is fast, reliable and requires minimal work-up. It can also be applied to the routine analysis of serum and urinary creatinine. The volatile derivative, which has not previously been described, is much more stable than the trimethylsilyl derivative and its mass spectrum displays no interfering peaks since naturally occurring fluorine has only one isotopic form.

EXPERIMENTAL

Materials and reagents

Unless otherwise stated, all reagents were AnalaR grade (BDH, Poole, Great Britain).

Ion-exchange procedure. Cation-exchange resin AG 50W-X12 (H^+), 200—400 mesh (Bio-Rad Labs, Richmond, Calif., U.S.A.).

Adsorption buffer: this buffer (pH 3.0) contained citric acid (40 mmoles/l) and disodium hydrogen orthophosphate (20 mmoles/l).

Elution reagents: sodium acetate, 0.1 and 0.5 mole/l; ammonium acetate, 0.1 and 0.5 mole/l.

Sodium hydroxide solution: 2.5 moles/l.

Lowry protein estimations. Alkaline tartrate reagent: this was prepared by dissolving sodium carbonate (20 g) and sodium or potassium tartrate (0.5 g) in 1l of sodium hydroxide solution (0.1 mole/l).

Copper sulphate solution: 4 mmoles/l.

Working alkaline copper reagent: this was prepared freshly each day by mixing alkaline tartrate reagent (45 ml) with copper sulphate solution (5.0 ml).

Folin—Ciocalteu reagent: Folin and Ciocalteu's phenol reagent (BDH) was diluted 4-fold with distilled water.

Standard protein solution: bovine serum albumin 100, 200, 300, 400, and 500 mg/l in distilled water.

Control serum. Wellcomtrol 3 (Wellcome Reagents, Beckenham, Great Britain) stated to contain 0.71 mmole creatinine per litre was used as control serum.

Creatinine determination by the Jaffe reaction. Sodium hydroxide: 0.5 and 1.5 moles/l.

Saturated picric acid: about 14 g/l.

Standard creatinine solutions: 0.443, 0.885, 1.33 and 1.77 mmoles/l in distilled water.

Instruments. The following instruments were used: a Pye-Unicam SP 1800 ultraviolet—visible spectrophotometer, a Perkin-Elmer Model 577 grating

infrared spectrophotometer, a Pye-Unicam Series 106 gas chromatograph fitted with a flame ionization detector (FID), a Varian MAT-731 high-resolution mass spectrometer and a Waters Assoc. Model ALC - GPC 204 liquid chromatograph comprising of a M6000A solvent delivery system a U6K universal injector and a Model 440 absorbance detector fixed at 254 nm.

Isolation of creatinine from serum and urine by cation-exchange chromatography

Polypropylene pipette tipes (Oxford Macro-set, Boehringer London, Lewes, Great Britain) were plugged with non-absorbant cotton wool and used as semi-micro chromatography columns. Aliquots of the resin (100 mg) were suspended in portions (2.5 ml) of sodium hydroxide solution, transferred to the columns, and allowed to drain briefly. Each column was then washed with water (2.0 ml), followed by adsorption buffer (4.0 ml).

The sample (0.5 ml) of serum or of urine (diluted 50 fold with water), standard creatinine solution, or water as a reagent and a column blank, was mixed with adsorption buffer (5.0 ml) and 5.0 ml of each diluted sample allowed to drain through a prepared column. Following a wash with distilled water (4.0 ml), the adsorbed creatinine was eluted with the appropriate eluting reagent (3.0 ml).

Measurement of protein concentration in eluates

To monitor the elution of protein from the ion-exchange columns, a control serum was processed according to the above procedure. Fractions (0.5 ml) were collected during the water wash and sodium acetate (0.5 mole/l) elution steps, and analysed as follows. Portions (0.3 ml) of each fraction or of standard protein solutions and of distilled water (to serve as blank) were added to working alkaline copper reagent (2.0 ml), mixed, and kept at room temperature for 5 min. Folin-Ciocalteu reagent (0.2 ml) was then added and the reaction mixtures were kept for a further 30 min at room temperature for colour development. The absorbances of the test and standard solutions were measured against the blank at 720 nm.

Elution of creatinine from the cation-exchange column

To monitor the elution of creatinine from the columns, an aqueous creatinine solution (0.885 mmole/l), a control serum (Wellcontrol 3) and distilled water (as a reagent and column blank) were each taken through the cation-exchange procedure described above. During the elution of creatinine with sodium acetate solution (0.5 mole/l), fractions (0.5 ml) were collected and analysed using the Jaffe reagents as follows. Portions (0.2 ml) were added to sodium hydroxide solution (1.0 ml); picric acid solution (0.2 ml) was then added, and the reaction mixtures incubated at room temperature for 30 min. The absorbances of the test solutions were measured at 490 nm against the column blank.

The elution of creatinine with ammonium acetate solutions ranging in concentration from 0.1 to 0.5 mole/l was also investigated. The Jaffe reac-

tion could not be used in this instance because of interference from ammonium ions; consequently creatinine was monitored in portions (0.5 ml) of the ammonium acetate eluates (diluted to 3.0 ml with water) by direct measurement of absorbance at 235 nm (the absorption maximum of creatinine at pH 7.0) against an appropriate blank.

Recovery experiments

The recovery of creatinine which had been added to a control serum, a pooled patients' serum, ultrafiltered serum, or urine (diluted 50 fold) was determined by diluting portions of each of these specimens (a) with an equal volume of a standard solution of creatinine (0.885 mmole/l) and (b) with an equal volume of water. Creatinine was then isolated from the samples by the ion-exchange procedure, and measured by the Jaffe reaction which was carried out as follows. A portion of each eluate (1.0 ml) was added to sodium hydroxide solution (1.5 moles/l, 1.0 ml) followed by picric acid solution (0.5 ml), the procedure was calibrated by treated creatinine standards similarly, with absorbance measurements made at 490 nm against a blank as before. The recoveries were estimated from the results so obtained.

High-performance liquid chromatography

The separation was carried out on a reversed-phase column (30 cm × 4 mm I.D.) consisting of a monomolecular layer of octadecyltrichlorosilane chemically bonded to 10 μm silica (μBondapak C_{18}, Waters Assoc., Milford, Mass., U.S.A.). Ammonium acetate solution (0.01 mol/l) was used as the mobile phase and elution was at 1 ml/min with a pressure drop of 1000 p.s.i.

For the isolation of serum and urinary creatinine, the specimens were pre-purified by the ion-exchange procedure described above. The eluate (3 ml) was then evaporated to dryness with a rotary evaporator. The residue was redissolved in 100 μl of ammonium acetate solution (0.01 mole/l) and 50 μl of the solution were injected into the chromatograph.

The peak corresponding to creatinine was collected and the solvent evaporated off as before.

Preparation and gas chromatography of O-trifluoroacetylcreatinine

The creatinine obtained by HPLC was treated with 100 μl of trifluoroacetic anhydride (TFAA) and heated for 5 min at 60°. After cooling, 2 μl of the clear solution were injected into the gas chromatograph. The column used (3.25 × 2.5 mm I.D.) was packed with 3% OV-210 on Chromosorb W, 80—100 mesh. The injector and oven temperatures were set at 200° and 130°, respectively.

This compound could be isolated as an oil by heating creatinine with TFAA and subsequent removal of excess TFAA and the trifluoroacetic acid formed with a rotary evaporator.

Preparation of creatinine trifluoroacetate

Creatinine (1 mg) was dissolved in trifluoroacetic acid (2 ml), and diethyl ether (5 ml) was added slowly with stirring until no more crystals were formed.

The crystals were filtered, washed with diethyl ether and recrystallised from choloroform—methanol (m.p. 155—156°).

Infrared spectroscopy

The spectrum of O-trifluoroacetylcreatinine was recorded as a thin film, and those of creatinine and creatinine trifluoroacetate as Nujol mulls. Potassium bromide prisms were used throughout.

Mass spectrometry

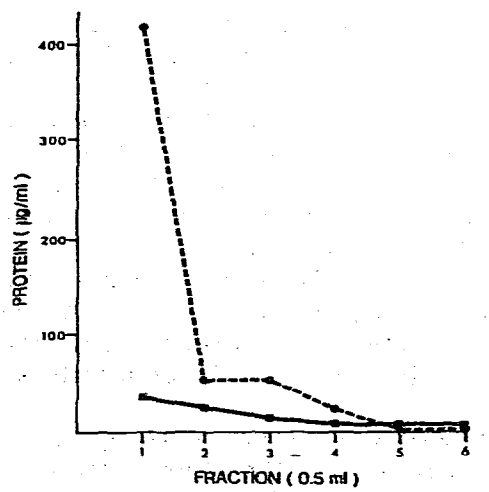
The mass spectrum of O-trifluoroacetylcreatinine was measured by injecting a sample in trifluoroacetic anhydride on to a gas chromatographic column (3.25 × 2.5 I.D.) packed with Chromosorb W, 80—100 mesh coated with 3% OV-210 coupled to the mass spectrometer. The source temperature was 200°.

RESULTS AND DISCUSSION

Isolation of creatinine by ion-exchange chromatography

When serum samples were applied to the cation-exchange columns in the manner described, only 0.1% of the protein present in the sample adsorbed to the column. Furthermore, as indicated by the elution profiles in Fig. 1, 90% of this adsorbed protein was eluted in the water wash. Consequently the final eluate, containing the desorbed creatinine, contained only 0.01% of the protein originally applied to the column.

As shown in Fig. 2, creatinine which had been adsorbed to the column from serum or aqueous solutions of creatinine was eluted (99 ± 1%) with



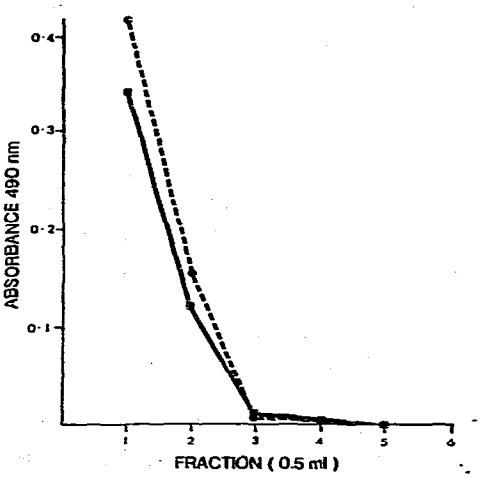


Fig.1. Elution of protein from cation-exchange column. —, protein in sodium acetate eluate; - - - -, protein elution during water wash.

Fig.2. Elution of creatinine from cation-exchange column. —, control serum; - - - -, creatinine standard.

2.5 ml of sodium acetate solution (0.1 mol/l). Similar results were obtained when ammonium acetate (0.1 mol/l) was used as the eluent.

Recoveries of creatinine which had been added to a control serum, a pooled patients' serum, ultrafiltered serum and urine (diluted 50 fold) when isolated by the cation-exchange procedure and assayed by the Jaffe reaction, were found to be 99, 98, 100 and 99%, respectively.

These results indicate that the isolation procedure was quantitative. It was considered that no significant advantage would be gained by including an ultrafiltration step [14] prior to the ion-exchange procedure since the small amount of protein remaining in the final eluate did not interfere with the subsequent HPLC isolation.

Ammonium acetate or sodium acetate eluents were selected for this study since it was considered that the strongly alkaline nature of the eluting buffers used by previous workers [7,8] could conceivably hydrolyse creatinine to creatine. Ammonium acetate is favoured as the eluent in the primary isolation procedure as this solvent proved to be satisfactory for further purification of the isolated creatinine by HPLC.

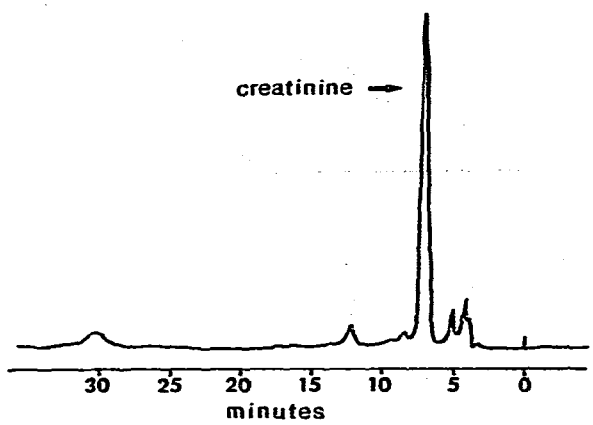
High-performance liquid chromatography

A number of solvent systems were tested before 0.01 *M* ammonium acetate solution was finally selected as the one most suitable for the separation of creatinine from biological fluids. For example, reversed-phase chromatography on a μBondapak C_{18} column with 0.005 *M* formic acid—methanol (25:1, v/v) and ion-pair chromatography with 10% methanol in PLC-B7 (containing 1-heptanesulphonic acid buffered at pH 3.5, Waters Assoc.) both gave excellent separation of synthetic creatinine standard. Unfortunately these systems proved to be unsuitable for the analysis of serum creatinine due to interfering substances from serum. Reversed-phase chromatography with ammonium acetate solution as the mobile phase effectively resolved creatinine from the interfering substances present in serum and in urine which had been observed with the other solvent systems. The concentration of the ammonium acetate solution seemed to have little influence on the retention of creatinine; thus 0.05 and 0.01 *M* solution gave virtually the same retention times, i.e. 7.5 min. It is clearly advantageous if the retention time does not vary much with slight changes in solvent concentration, since highly reproducible results can then be expected. This was shown by the analysis of over 50 serum specimens for creatinine without any alteration of retention time. Figs. 3 and 4 show typical separations of serum and urinary creatinine, respectively; it is apparent from the chromatograms that urine samples generally contained fewer interfering compounds.

The sensitivity of the UV detector allowed easy detection of 10 ng of creatinine at 0.005 a.u.f.s. without baseline drift. It was therefore possible to detect the creatinine present in 100 μl samples of normal serum.

Preparation and characterization of O-trifluoroacetylcreatinine

Since creatinine can exist in the enol form (Fig. 5), acylation of the molecule is possible. The required derivative was simply prepared by reacting creatinine with hot TFAA. The product, O-trifluoroacetylcreatinine, was



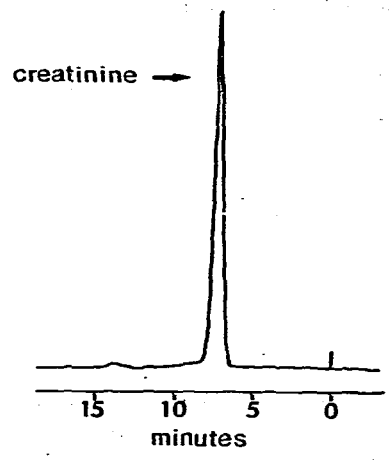


Fig.3. HPLC separation of serum creatinine.

Fig.4. HPLC separation of urinary creatinine.

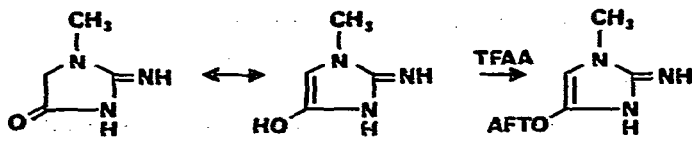


Fig.5. O-Trifluoroacylation of creatinine.

characterized by infrared (IR) spectroscopy and gas chromatography—mass spectrometry with the following results: in the IR spectra, the carbonyl absorption of creatinine at 1670 cm^{-1} was shifted to higher frequency at 1775 cm^{-1} indicating O-acylation (Fig. 6); gas chromatography on 3% OV-210 gave a single peak which, when monitored with the mass spectrometer, gave a molecular ion at $M^+ = 209$ confirming trifluoroacylation. Peaks at m/e = 140 ($M^+ - CF_3$) and m/e — 112 ($M^+ - COCF_3$) were also consistent with trifluoroacylation (Fig. 7). As expected from the occurrence of fluorine in only one isotopic form, it was satisfactory to find that the mass spectrum contained no peaks which might cause difficulties in isotope dilution work using [$^{15}N_3$]creatinine.

Further trifluoroacylation of the derivative was not observed. This is to be expected since after O-trifluoroacylation, hydrogen bonding of a fluorine atom of the secondary amine group of the molecule is possible (Fig. 8) and this effectively prevents further substitution. Creatinine isolated from serum and urine samples by HPLC gave the same derivative when treated with TFAA, so confirming the identity and homogeneity of the isolated compound.

O-Trifluoroacetylcreatinine was found to be stable if kept free from moisture, and was much more easily handled then tris(trimethylsilyl)creatinine. In the presence of moisture, as in "wet" organic solvents, colourless needles slowly crystallised out solutions of O-trifluoroacetylcreatinine. In the IR

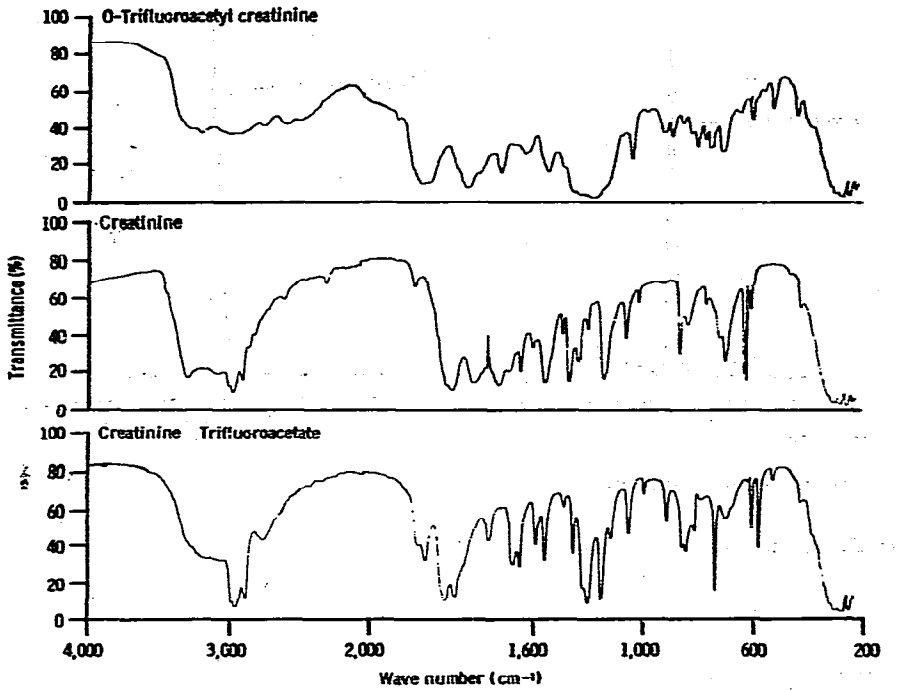


Fig.6. IR spectra of O-trifluoroacetylcreatinine, creatinine and creatinine trifluoroacetate.

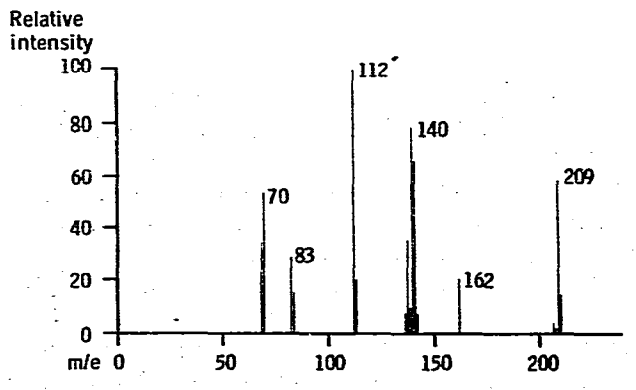


Fig.7. Mass spectrum of O-trifluoroacetylcreatinine.

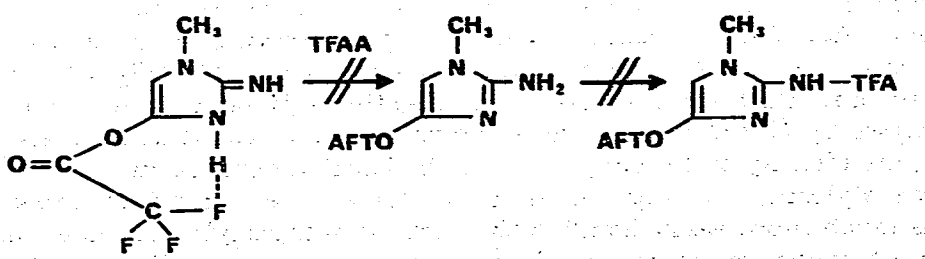


Fig.8. Scheme showing how further substitution of creatinine is prevented after O-trifluoroacylation.

spectrum of the crystalline product (Fig. 6), the carbonyl absorption of creatinine was shifted to 1710 cm^{-1}; another carbonyl peak at 1775 cm^{-1} could be assigned to trifluoroacetic acid, since a broad hydroxyl absorption in the 3000 cm^{-1} region was typical of an acid. The structure of this product was therefore considered to be that of creatinine trifluoroacetate. This was confirmed by its conversion black to O-trifluoroacetylcreatinine by heating with TFAA and by the preparation of an identical substance on reacting pure creatinine with trifluoroacetic acid.

CONCLUSIONS

The objectives of this work were to develop an efficient method for the separation and purification of creatinine from biological fluids and a practicable means of derivatizing creatinine so that it could be characterized by gas chromatography—mass spectrometry. Both of these objectives have been realized, so that a definitive method of assay of creatinine in biological fluids is now feasible.

Successive cation-exchange chromatography and reversed-phase HPLC with UV detection offers a rapid (30 min) clean-up procedure suitable for both serum and urine. The sensitivity (detection limit 5 ng) is such that a 100-μl sample of normal serum, can be processed satisfactory. O-trifluoroacetylcreatinine is readily prepared and easily characterized by gas chromatography—mass spectrometry.

The speed and simplicity of the purification procedure suggest that by the addition of internal standard to the original specimen it would be possible to develop a reference method of assay of creatinine.

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