Journal of Chromatography, 459 (1988) 245–249
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1456

DETERMINATION OF SALSOLINOL BY ION-EXCHANGE CHROMATOGRAPHY WITH GLYCYLGLYCINE AS THE POST-DERIVATIZING AGENT

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SUMMARY

The determination of salsolinol in human urine was carried out by ion-exchange chromatography on two coupled columns of a weakly acidic ion exchanger with a hydrophilic matrix (Asahipak ES-502C). Salsolinol was first isolated from urine by adsorption on Amberlite CG-50. It was eluted together with catecholamines by 2/3 *M* boric acid solution. The amines were then separated by isocratic elution from the first column of Asahipak with 0.05 *M* sodium succinate buffer (pH 5.5) containing 0.015 *M* borate and 0.5 m*M* ethylenediaminetetraacetate. Epinephrine, norepinephrine, dopamine and salsolinol were eluted in that order. The salsolinol-containing fraction was then transferred, by column switching, to a second Asahipak column and eluted with the same mobile phase. Salsolinol was determined fluorimetrically by reaction with glycylglycine in the presence of hexacyanoferrate(III) at pH 7.5–8 and 65°C. Samples could be analysed every 47 min. The detection limit for salsolinol was 2 pmol/ml.

INTRODUCTION

Salsolinol (1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline) has been found in urine[1], cerebrospinal fluid[2,3], rat and human brain tissue[4–6], and food and beverage samples[7]. The determination of salsolinol in these samples involved extraction by ion exchange[8] or alumina adsorption[1,7,9] and analysis by gas chromatography–mass spectrometry[7,9] or a radioenzymatic method[10]. Ion-pair reversed-phase liquid chromatography with electrochemical detection was also effective for the determination of salsolinol in human urine[8].

We have found that salsolinol reacts rapidly with glycylglycine at elevated temperatures and at pH 7.5–8 in the presence of potassium hexacyanoferrate(III) to give fluorescent compound(s), and that salsolinol could be separated from catecholamines on a column of hydrophilic cation-exchange resin (Asahipak ES-502C) by elution

0021-9673/88/$03.50

with a 0.05 *M* succinate buffer (pH 5.5) containing 0.015 *M* borate and 0.5 m*M* ethylenediaminetetraacetate.

These findings were applied to the determination of salsolinol in human urine by combining ion-exchange extraction, separation of the salsolinol fraction on two coupled columns of Asahipak ES-502C by column switching and fluorimetric determination of salsolinol in the eluate with glycylglycine as the post-derivatizing agent.

EXPERIMENTAL

Materials

Epinephrine bitartrate, norepinephrine bitartrate, dopamine hydrochloride and salsolinol were purchased from Sigma (St. Louis, MO, U.S.A.) and glycylglycine from Nakarai Chemicals (Kyoto, Japan). Other chemicals were of analytical-reagent grade from Yashima Pharmaceutical (Osaka, Japan). Stock solutions of amines (1 m*M*) were prepared in 0.01 *M* hydrochloric acid, and were diluted in 0.04 *M* succinate–0.4 *M* borate buffer (pH 5.3) containing 1 m*M* ethylenediaminetetraacetate to give standard solutions of various concentrations.

A constant-flow pump (Model Trirotar III and V; Jasco, Tokyo, Japan) was used to pump buffer through the chromatographic columns. A dual-head pump (Model SP-024-2; Jasco) was used to pump reagents and mix them with the elutate. A fluorimeter (Model FP-115; Jasco) equipped with a 30-μl flow cell was used to measure fluorescence. Samples were injected by an automatic injector (Model KSST-60J; Kyowa Seimitsu, Tokyo, Japan). A four-way automatic valve (Sanuki Kohgyoh, Tokyo, Japan) was used to connect two columns of Asahipak ES-502C in series.

Methods

Amberlite CG-50 was buffered and equilibrated with a 0.4 *M* phosphate buffer (pH 6.5) as described previously[11]. The buffered resin was poured into a tube (18 × 0.5 cm I.D. with a 20-ml reservoir) with 0.4 *M* phosphate buffer (pH 6.5) and allowed to settle under gravity to form a resin bed 12 cm high. The column was washed with 2 ml of water before use.

A 10.0-ml portion of filtered urine was mixed with 1.0 ml of a 2% solution of semicarbazide hydrochloride containing 0.5% each of sodium metabisulphite and disodium ethylenediaminetetraacetate dihydrate, and the mixture was adjusted to pH 6.3–6.4 with 0.5 *M* sodium hydrogencarbonate solution. The mixture was then applied to the Amberlite CG-50 column, which was first washed with 6 ml of deionized water and then with 2 ml of 2/3 *M* boric acid solution. A further 3 ml of boric acid solution were used to elute salsolinol from the column into a test-tube containing 1.95 ml of 0.1 *M* succinic acid solution and 0.05 ml of 0.1 *M* ethylenediaminetetraacetate solution containing 5% β-thiodiglycol. The salsolinol fraction was stored in a refrigerator.

A 0.3-ml of aliquot of this fraction was injected into the first column of Asahipak ES-502C (10 × 0.76 cm I.D.), kept at 35°C. The mobile phase (0.05 *M* succinate–0.015 *M* borate–0.5 m*M* ethylenediaminetetraacetate, pH 5.5) was pumped at a rate of 1.5 ml/min and the salsolinol fraction from the first column was switched to the second column of Asahipak ES-502C (10 × 0.76 cm I.D.), connected to the first column and kept at 60°C, and eluted with the same mobile phase at a flow-rate of 1.5

ml/min. The time to rotate the automatic valve in order to transfer the salsolinol fraction from the first to the second column was found by monitoring fluorimetrically the elution of salsolinol from the first column. When the timing of the rotation of the automatic valve is correct, the salsolinol peak in the elution pattern disappears.

The eluate from the second column was mixed with an equal mixture of reagents A and B; reagent A was a solution of 0.1 *M* glycylglycine (pH 6.5) containing 0.2 *M* boric acid and 0.05 *M* tartaric acid and reagent B was 0.25 *M* potassium borate buffer of pH 9.4 containing hexacyanoferrate(III) (0.01%, w/v). Each reagent was pumped at a flow-rate of 0.47 ml/min with a dual-head pump and mixed by using a T-shaped connector. The mixture of reagents was filtered through a guard column (15 × 0.6 cm I.D.) packed with 5-μm Shodex DS-613 polystyrene gel (Showa Denko, Tokyo, Japan) to remove fine particles that would cause background noise when passing through the flow cell. The filtered reagent mixture was mixed with the eluate, using a T-shaped connector and heated in a PTFE tube (50 m × 0.5 mm I.D.), immersed in a water-bath kept at 65°C. The fluorescence was measured with a fluorimeter using a UV-D36C as excitation light filter (transmittance maximum 365 nm) and a Y-44 as emission light filter (transparent to visible light of wavelength longer than 420 nm). Both filters were of coloured glass manufactured by Toshiba (Tokyo, Japan). A diagram of the equipment is shown in Fig. 1.

RESULTS AND DISCUSSION

Salsolinol was measured fluorimetrically by reaction with glycylglycine in the presence of hexacyanoferrate(III). This reaction is apparently analogous to that of glycylglycine with oxidized catecholamines. *e.g.*, adrenochrome[12]. The optimal pH for this reaction is 7.5–8 when performed at 65°C in a PTFE tube (50 m × 0.5 mm I.D.). The excitation and emission maxima were 360 and 490 nm, respectively.

The salsolinol fraction obtained by an ion-exchange method using Amberlite CG-50 (buffered at pH 6.5) as the adsorbent contains catecholamines. Therefore, a chromatographic system was developed which uses Asahipak ES-502C, a cross-linked vinyl–alcohol copolymer with carboxymethyl groups, as the stationary phase and

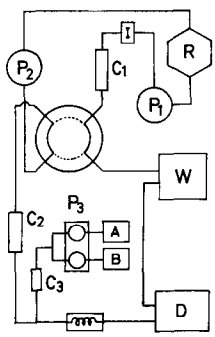

Fig. 1. Diagram of the column switching equipment. R, mobile phase reservoir; I, automatic injector; P_1, Trirotar III; P_2, Trirotar V; P_3, dual-head pump (Model SP-024-2, Jasco); C_1 and C_2, Asahipak ES-502C columns; C_3, guard column; D, fluorimeter (Model FP-115, Jasco); W, waste; A, reagent A reservoir; B, reagent B reservoir.

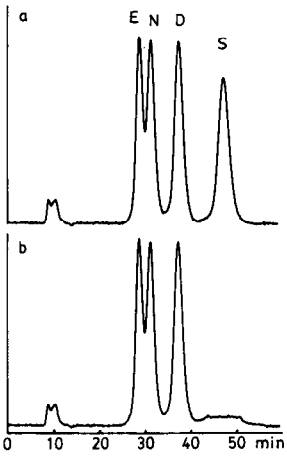

Fig. 2. Elution of standard samples from the first column. Amines in the elutate were monitored fluorimetrically. (a) Separation of epinephrine (E, 20 pmol/ml), norepinephrine (N, 20 pmol/ml), dopamine (D, 100 pmol/ml) and salsolinol (S, 50 pmol/ml); (b) after transfer of the salsolinol peak to the second column.

0.05 *M* succinate buffer (pH 5.5)–0.015 *M* borate–0.5 m*M* ethylenediaminetetraacetate as the mobile phase (Fig. 2a). Elution was performed at 35°C, and in order to separate impurities that would be eluted with salsolinol under these conditions the salsolinol peak from the first column was transferred to the second column of Asahipak ES-502C by using a four-way valve. The first column was connected to the second 36 min after sample injection, and 7.5 min later the columns were disconnected. The salsolinol peak disappeared from the elution pattern of the first column as shown in Fig. 2b. The salsolinol peak, transferred to the second column, was eluted with the same buffer as that used for the first column. The temperature of the second

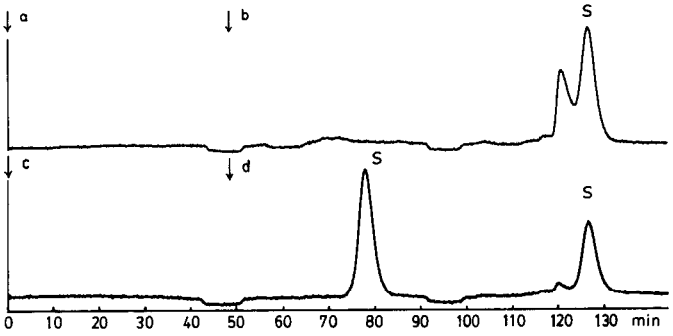

Fig. 3. Elution of a standard and urine samples transferred from the first to the second column. Arrows indicate the time of injection of a sample into the first column. The upper and lower curves represent different set of experiments. Standard salsolinol (50 pmol/ml, the first peak on the lower curve) was eluted from the second column 78 min after injection (at arrow c) into the first column. A urine sample was injected at d and 78 min after injection the salsolinol peak was eluted after a small impurity peak. A urine sample injected at a yielded no peak 78 min after injection, and the urine sample injected at b yielded a peak 78 min after injection (peak S on the upper curve).

column was 60°C, and the difference in temperature between the first and the second columns made it possible to separate salsolinol from impurities. The impurities were eluted faster than salsolinol, as shown in Fig. 3. Use of the same mobile phase for both stages of the HPLC separation (using two columns of Asahipak ES-502C) yielded a relatively stable baseline and a limit of detection of 2 pmol/ml for salsolinol.

A linear relationship between the peak height and the concentration of salsolinol added to the column was obtained between 5 and 200 pmol/ml. Samples could be analysed every 47 min. When salsolinol was added to a urine sample at a concentration of 10 pmol/ml, the mean recovery was 95 $\pm$ 3.2% (C.V., $n = 6$). When the concentration of free salsolinol was measured in normal human urine samples, the range of concentration was 0–30 pmol/ml. These values agree with the value of free salsolinol in the urine pool (11.2 $\pm$ 0.95 pmol/ml, $n = 10$) reported by Sjöquist and Ljungquist[9].

The results indicate that this method is suitable for the determination of salsolinol in human urine and other biological samples.

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