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ANALYSIS OF URINARY CATECHOLAMINES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE PRESENCE OF LABETALOL METABOLITES

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SUMMARY

Common sample preparation methods for catecholamines lead to contamination with metabolites of labetalol, an anti-hypertensive drug. When the extracts are analyzed by cation-exchange high-performance liquid chromatography, these metabolites are separated from the catecholamines, but their strong retention lengthens the analysis time. A procedure has been developed for complete removal of these drug metabolites from acidified urine by the use of XAD-4 resin. Loss of catecholamines is monitored by an internal standard. This pretreatment can be combined with extraction by weak cation-exchange resin and borate elution to simplify catecholamine analysis for patients receiving labetalol.

INTRODUCTION

Labetalol, a specific competitive antagonist of both α- and β-receptors, has found widespread clinical use for the treatment of hypertension. Its interference in the determination of urinary catecholamines, based on cation-exchange extraction and fluorometry, has been described[1,2]. Recently, interference by labetalol metabolites was reported for ion pair high-performance liquid chromatography (HPLC) determinations of plasma catecholamines, based on alumina extraction[3]. The metabolite that causes interference has not been identified[4]. Earlier studies have shown that the major metabolites of labetalol are glucuronides[5]. Labetalol itself was not extracted from plasma by alumina[3] or from urine by cation-exchange chromatography[6].

In a previous investigation the major urinary metabolites of labetalol were resolved by ion pair chromatography on a C_{18} column[6]. Ultraviolet (UV), fluorescence, or electrochemical detection (ED) was employed. Only 1–2 metabolite peaks could be observed under these conditions. A recent report on HPLC of labetalol in urine did not mention any metabolites[7].

Cation-exchange chromatography has been applied to the analysis of catecholamines in plasma and urine[8,9]. When drugs are analyzed on a weak cation-exchange silica column, hydrophobic amines with polynuclear structures (such as prazosin) are retained more strongly than smaller monocyclic compounds (such as iso-

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proterenol)[10]. Labetalol and its metabolites, having two rings, should be retained longer than the catecholamines under cation-exchange conditions.

The purpose of this investigation was to separate labetalol metabolites by weak cation-exchange and reversed-phase HPLC. Further, a sample cleanup was devised to remove the labetalol metabolites from urine to facilitate quantitation of urinary catecholamines.

EXPERIMENTAL

Materials

Labetalol, epinephrine, norepinephrine, and the internal standard, N-methyldopamine (NMD), were purchased from Sigma (St. Louis, MO, U.S.A.). HPLC-grade potassium dihydrogen phosphate, acetonitrile, and methanol were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.). Water for mobile phases was purified by ion-exchange and glass distillation. The other reagents and solutions used were analytical grade. SM-4 beads (XAD-4), 20–50 mesh, were from Bio-Rad Labs. (Richmond, CA, U.S.A.). They were ground in an aqueous slurry in a stainless-steel mill, and the product was sized with mesh screens to isolate 80–120 mesh material. This fraction was washed four times with methanol, dried by vacuum filtration, and stored in a tightly closed container at room temperature. XAD-4, 0.1–0.2 mm; research-grade, purchased from Serva Biochemicals (Westbury, NY, U.S.A.), gave results equivalent to the material we prepared. Prepacked cation-exchange columns, containing Bio-Rex® 70, were from Bio-Rad Labs.

HPLC instrumentation

The chromatographic system (Bio-Rad Labs.) was composed of a Model 1330 dual-piston pump, a Model 1305A variable-wavelength (UV) detector, or a Model 1340 electrochemical detector, a Model 450 column heater, a Model 1725 injector with a 20-μl sample loop, and a Model 1321 recorder. A 150 × 4.6 mm I.D., 5-μm ODS column (Bio-Rad Labs.) with a 30 × 2.1 mm I.D. guard cartridge was used for reversed-phase chromatography. A 150 × 4.6 mm I.D., 5-μm carboxymethyl (weak cation-exchange) column (Bio-Rad Labs.) with a 30 × 2.1 mm I.D. reversed-phase guard cartridge was used for cation-exchange chromatography.

Mobile phase preparation

For catecholamine analysis, 8.2 g of potassium dihydrogen phosphate was dissolved in 950 ml water and adjusted to pH 5.20 with 0.5 *M* sodium hydroxide; 50 ml methanol was added, and the mixture was filtered under vacuum through a 0.45-μm membrane. The eluent for rapid cation-exchange HPLC of labetalol metabolites was prepared as follows: 4.60 g of ammonium dihydrogen phosphate and 4.20 g of citric acid monohydrate were combined with 850 ml water. The pH was adjusted to 6.0 with 2 *M* sodium hydroxide; 150 ml acetonitrile was added, and the mixture was filtered. The eluent for labetalol analysis by reversed-phase HPLC was prepared as follows: 6.8 g of potassium dihydrogen phosphate was dissolved in 800 ml water and adjusted to pH 2.70 with 2 *M* phosphoric acid; 200 ml acetonitrile was added and the mixture was filtered.

HPLC operating conditions

For routine cation-exchange HPLC of catecholamines, the guard cartridge and analytical column were maintained at 40°C with a flow-rate of 1.0 ml/min. The electrochemical detector was set at 0.55 V *vs.* Ag/AgCl reference electrode with a 5-s time constant and a gain of 5 nA/V full scale. For rapid cation-exchange analysis of labetalol metabolites, the same conditions were employed except the gain was changed to 50 nA/V full scale. For the detection of labetalol the voltage was raised to 0.90 V.

For reversed-phase HPLC, the guard cartridge and analytical column were maintained at 45°C with a flow-rate of 1.5 ml/min. The UV detector was set at 301 nm with 0.005 a.u.f.s. The electrochemical detector was set at 0.55 V with a 2-s time constant and a gain of 50 nA/V. For the detection of labetalol the voltage was raised to 1.00 V.

Urine sample preparation for catecholamine analysis

Collections of urine (24-h samples) were preserved with 6 *M* hydrochloric acid (10 ml/l). The prepacked cation-exchange column was inverted to resuspend the resin, opened, and allowed to drain. Urine (3.0 ml) was combined with 0.2 ml NMD (5 mg/l) and 5 ml 0.03 *M* ammonium acetate buffer containing 0.1% EDTA. The pH was brought into the range 6.0–7.0 with 0.5 *M* sodium hydroxide. This mixture was poured onto the column and allowed to drain. The column was rinsed with 5 ml of the acetate buffer, then the catecholamines were eluted with 6 ml 2% ammonium pentaborate. The eluates were brought to pH 5.0–5.5 with 0.3 ml 1 *M* acetic acid.

Urine purification with XAD-4 resin

A 300-mg amount of XAD-4 resin was transferred to a 150 × 16 mm test tube, suspended in 10 ml methanol, and centrifuged 10 min at 1000 *g*. The supernatant was removed, and washing was repeated with 0.05 *M* hydrochloric acid and then with water. After adjustment of urine (6.0 ml) and 0.4 ml NMD (5 mg/l) to pH 2.0 with 1 *M* hydrochloric acid or 0.5 M sodium hydroxide, the sample was poured onto the resin. The solution was mixed for 60 s and then centrifuged for 10 min at 1000 *g*. The supernatant was injected directly for labetalol analysis, or 3.2 ml was transferred to another tube and treated as described above for catecholamine analysis.

RESULTS AND DISCUSSION

Sample preparation and HPLC analysis of urine catecholamines followed the instructions supplied for the Bio-Rad kit. Under the conditions used for weak cation-exchange HPLC, the retention times of epinephrine, norepinephrine, NMD and dopamine were 8.0, 9.2, 10.0 and 11.0 min, respectively (Fig. 1). Urines from patients receiving labetalol showed up to seven additional peaks with retention times ranging from 16 to 80 min (Fig. 1B). Although the quantitation of catecholamines in that sample was not affected, the analysis of subsequent samples was adversely affected by the late-eluted peaks. Removal of the guard cartridge had no significant effect on the elution times of the labetalol metabolites, demonstrating that the hydrophobic character of the carboxymethyl bonded phase was responsible for the strong retention observed.

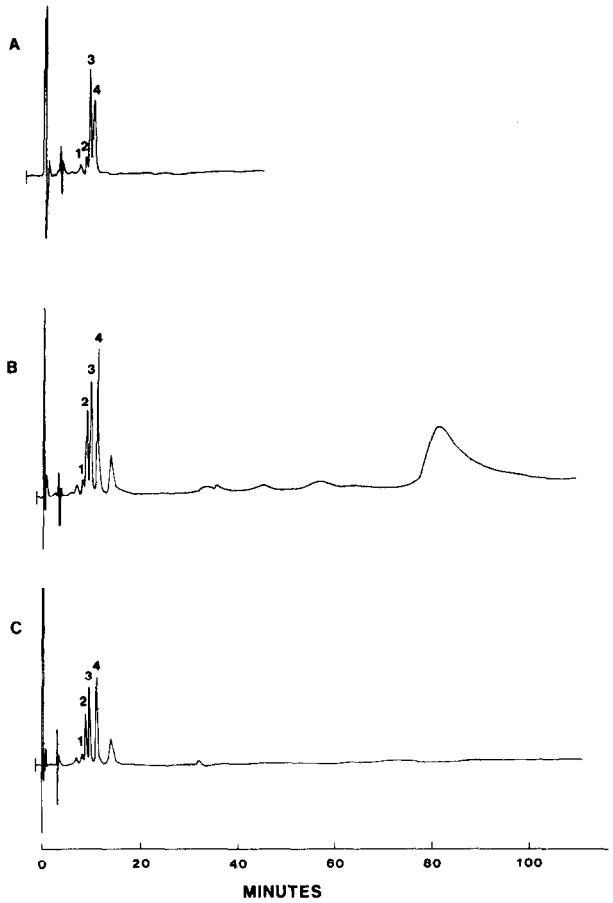

Fig. 1. Chromatograms of urinary catecholamines obtained with a cation-exchange column, mobile phase, 0.06 *M* phosphate buffer (pH 5.2) containing 5% methanol; flow-rate, 1.0 ml/min at 40°C; ED at 5 nA/V full scale and 0.55 V. (A) Drug-free urine; (B) urine containing labetalol metabolites after cation-exchange purification; (C) urine containing labetalol metabolites after XAD-4 pretreatment and cation-exchange purification. Peaks: 1 = epinephrine; 2 = norepinephrine; 3 = N-methyldopamine; 4 = dopamine.

In order to characterize the metabolite pattern by cation-exchange HPLC further, a mobile phase was prepared with 0.06 *M* citrate–phosphate buffer (pH 6.0) containing 15% acetonitrile, which permitted more rapid elution of the peaks. Under these conditions, the catecholamines and the labetalol metabolites were observed simultaneously and in less than 20 min (Fig. 2). The elution order was not affected by the mobile phase change, and the relative concentrations of different peaks were easily determined. Fifteen different urine samples, examined under these conditions, revealed similar patterns for the major and minor metabolites. A higher voltage potential was required for detection of labetalol than for its metabolites, in accordance with an earlier report[6].

Reversed-phase chromatography conditions were developed to facilitate the resolution of labetalol from its metabolites. A 0.05 *M* phosphate buffer at pH 2.7

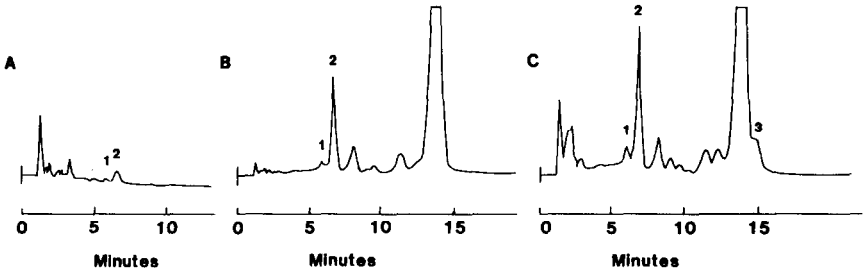

Fig. 2. Chromatograms of labetalol metabolites obtained with a cation-exchange column, mobile phase, 0.06 *M* citrate–phosphate buffer (pH 6.0) containing 15% acetonitrile; flow-rate, 1.0 ml/min at 40°C, ED at 50 nA/V full scale. (A) Drug-free urine, detector at 0.55 V; (B) urine containing labetalol metabolites, detector at 0.55 V; (C) urine containing labetalol metabolites, detector at 0.90 V. Peaks: 1 = norepinephrine; 2 = dopamine; 3 = labetalol.

was employed to obtain a good electrochemical response. The acetonitrile concentration was varied from 10 to 30% to determine optimal conditions for resolving the metabolites. Elution with 20% acetonitrile permitted analysis of labetalol and its metabolites in less than 20 min (Fig. 3). Under these conditions the catecholamines are eluted with the solvent front.

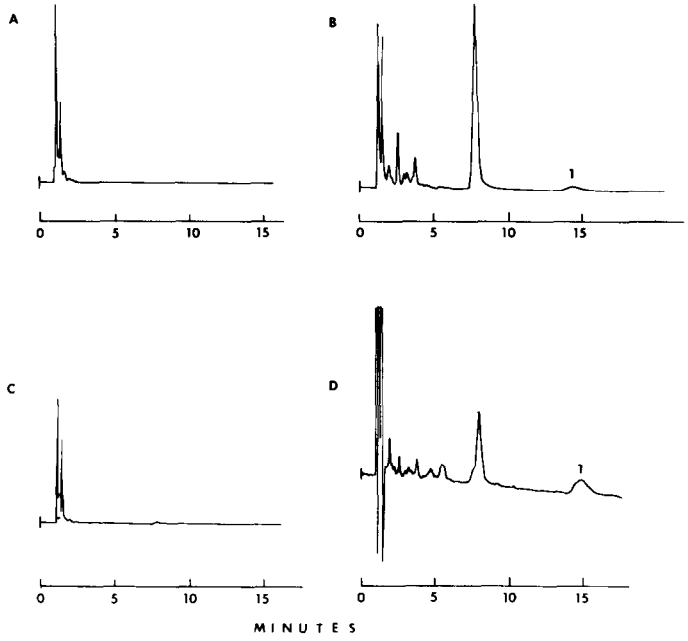

Fig. 3. Chromatograms of labetalol metabolites obtained with a reversed-phase column, mobile phase, 0.05 *M* phosphate buffer (pH 2.7) containing 20% acetonitrile; flow-rate, 1.5 ml/min at 45°C; ED at 100 nA/V full scale and 1.00 V. (A) Drug-free urine; (B) urine containing labetalol metabolites after cation-exchange purification; (C) urine containing labetalol metabolites after XAD-4 pretreatment and cation-exchange purification; (D) same sample as (B) but UV detection at 301 nm and 0.005 a.u.f.s. Peak 1 = labetalol.

Labetalol has a secondary absorption peak with a maximum at 301 nm. With reversed-phase chromatography it was possible to examine the labetalol metabolites after cation-exchange purification (Fig. 3D). These chromatograms served to confirm the identity of the peaks observed by ED. Since urine extracts from patients who took additional drugs did not reveal additional peaks, our sample preparation and detection methods are apparently quite selective. Labetalol was well separated from its metabolites under these conditions and was regularly detected in filtered urines, which were injected without any extraction.

XAD-4 resin is a low-polarity styrene–divinylbenzene copolymer, which possesses a high adsorptive capacity. It has been employed for removal of trace organic impurities in water[11] as well as for clinical applications, such as the extraction of metanephrines from plasma[12]. Ionic species show a maximum retention at their isoelectric pH. However, compounds which contain indole groups or long aliphatic side chains have been isolated from both acidic and neutral media[13]. Since labetalol metabolites are hydrophobic, it should be possible to adsorb them on XAD-4 at a low pH, where catecholamines are only weakly adsorbed.

Preliminary experiments with SM-4 beads, 20–50 mesh, showed incomplete removal of the metabolites from urine. When the ground material was used, adsorption was complete within 3 min; in fact, the urinary pigments were also removed by this technique. Examination of the treated urine by reversed-phase HPLC showed that the minor metabolites were absent and greater than 99% of the major metabolite was adsorbed (Fig. 3C). When these urines were examined after cation-exchange purification (Fig. 1C), the late-eluted peak was no longer observed. A peak which was eluted after dopamine was seen in urines that had been stored over 2 weeks at 4°C; it may represent a breakdown product resulting from slow *in vitro* hydrolysis. Catecholamines were also adsorbed on XAD-4, but this process was minimized by the acidic medium and the short mixing time. Since the internal standard was also adsorbed, it could be used to correct for the loss.

We developed standard curves by the addition of 50, 100, 200 and 400 μg/l in triplicate to normal urine. The samples were treated with XAD-4 and Bio-Rex 70, and results were compared both to urines which were treated with Bio-Rex 70 only and to dilutions of the catecholamines in mobile phase which were directly injected onto the HPLC column. Linear calibration curves were obtained for up to 200 μg/l for epinephrine and norepinephrine and up to 400 μg/l for dopamine; the correlation coefficient (r) was 0.9997 for each curve. After the two-step extraction the absolute recoveries for epinephrine, norepinephrine, dopamine, and the internal standard were 79%, 76%, 71%, and 67% respectively; after Bio-Rex 70 extraction alone, the recoveries were 85%, 83%, 85% and 76%.

CONCLUSION

In the ion pair reversed-phase chromatography of catecholamines, labetalol or one of its metabolites is eluted close to epinephrine. When urine extracts were chromatographed on a weak cation-exchange column or by reversed-phase chromatography without ion pairing, labetalol metabolites were found to be more hydrophobic than catecholamines. This observation led to the extraction of labetalol from urine with XAD-4, a hydrophobic macroreticular resin. This sample pretreatment selectively isolates catecholamines.

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REFERENCES

1 K. Kobayashi, Y. Miura, H. Tomioka, H. Sakuma, M. Adachi, K. Abe and K. Yoshinaga, *Tohoku J. Exp. Med.*, 126 (1978) 201.
2 L. Miano, R. Kolloch and V. DeQuattro, *Clin. Chim. Acta*, 95 (1979) 211.
3 P-M. G. Bouloux and D. Perrett, *Clin. Chim. Acta*, 150 (1985) 111.
4 D. Jack, *J. Cardiovasc. Pharmacol.*, 3 (suppl. 1) (1981) 572.
5 R. T. Brittain, D. M. Harris, D. Jack and D. A. Richards, in M. E. Goldberg (Editor), *Pharmacological and Biochemical Properties of Drug Substances*, Vol. 2, American Pharmaceutical Association, Washington, DC, 1979, p. 229.
6 L. E. Martin, P. Carey and R. Bland, *Methodol. Surv. Biochem.*, 7 (1978) 227.
7 J. Wang, M. Bonakdar and B. K. Deshmukh, *J. Chromatogr.*, 344 (1985) 412.
8 R. Hjelmdahl, *Am. J. Physiol.*, 247 (1984) E13.
9 S. R. Binder and M. A. Nelson, in *1985 Electroanalytical Symposium*, BAS Press, West Lafayette, IN, 1985, p. 24.
10 S. Binder and M. A. Nelson, 1985, unpublished work.
11 G. A. Junk, J. J. Richard, M. D. Grieser, D. Witiak, J. L. Witiak, M. D. Arguello, R. Vick, H. J. Svec, J. S. Fritz and G. V. Calder, *J. Chromatogr.*, 99 (1974) 745.
12 M.-T. Wang, M. Yoshioka, K. Imai and Z. Tamura, *Clin. Chim. Acta*, 63 (1975) 21.
13 G. C. Mills, *J. Chromatogr.*, 355 (1986) 193.