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Note

Validation of an amperometric high-performance liquid chromatographic determination of epinephrine in bupivacaine and epinephrine injection

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Epinephrine, (−)-3,4-dihydroxy-α-[(methylamino)methyl]benzyl alcohol, as the bitartrate salt at a 1:200 000 dilution is contained in the 0.5% bupivacaine · HCl, 2-piperidinecarboxamide, 1-butyl-N-(2,6-dimethylphenyl)-, monohydrochloride monohydrate, injecton in order to reduce the rate of absorption and peak plasma concentration of the local anesthetic[1]. This combination product available as Marcaine® · HCl with epinephrine 1:200 000 (Winthrop Pharmaceuticals, New York, NY, U.S.A.) is packaged in single-dose 30-ml ampules, 10-ml and 30-ml vials and multi-dose 50-ml vials. The development and validation of a sensitive and specific stability-indicating high-performance liquid chromatography (HPLC) assay method utilizing amperometric detection was desired as an improvement over and simplification of previous methods for epinephrine in this product.

Amperometric detection has been used for the HPLC determination of the non-catechols chlorpromazine[2] and acetaminophen[3] in plasma with oxidation at glassy carbon electrodes at +0.85 and +0.80 V *vs.* Ag/AgCl respectively. Quantitation of non-catechol drugs in dosage forms as well using amperometric detection has been studied. These studies have included *cis*-platinum[4] and naloxone[5] injections and atropine in tablets and tinctures[6].

Epinephrine has been measured in dosage forms previously by non-electrochemical methods such as specific rotation and spectrophotometry. The method for epinephrine in "Bupivacaine and epinephrine injection" found in the United States Pharmacopoeia (U.S.P.) XXI is spectrophotometric with detection at 490 nm following an oxidation with potassium ferricyanide[7] while that for epinephrine in "Epinephrine bitartrate inhalation aerosol" is also spectrophotometric, measured at 530 nm following a ferrocitrate reaction[8]. An UV spectrophotometric method measured at 280 nm is applied to "Epinephrine ophthalmic solution"[9], whereas the specific rotation method is currently official for "Epinephrine nasal solution"[10]. An HPLC method with UV detection at 254 nm has been applied to "Epinephrine injecton", U.S.P., whereas electrochemical detection (ED) with oxidation at +0.70 V was used for an impurity analysis in that study[11].

The catechol functionality of epinephrine makes its detection amenable to oxidative amperometry at relatively low positive voltages. This, coupled with the

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extreme sensitivity of amperometric detection has been successfully applied to innumerable studies using HPLC separations of catechols, catecholamines and their metabolites from biological tissues and fluids. A recent literature sampling include measurements from plasma and urine[12–17].

Several previous HPLC assay methods for epinephrine in dosage forms have included amperometric detection. Epinephrine in "Lidocaine and epinephrine injection" has been measured by two alternate amperometric HPLC methods with oxidation at +0.90 and +0.65 V, respectively[18,19]. Similarly, epinephrine in "Epinephrine injection"[10] and in "Prilocaine and epinephrine injection"[20] was determined by HPLC–ED at +0.65 V. The optimization and validation of an amperometric HPLC method for epinephrine analysis in "Bupivacaine and epinephrine injection" appearing in the seventh supplement to U.S.P. XXI[21] is described in the present study.

EXPERIMENTAL

Reagents

Methanol from Fisher Scientific and water (Sybron/Barnstead) were HPLC grade. Sodium phosphate monobasic monohydrate, ethylenediaminetetraacetic acid disodium salt, 85% phosphoric acid, sodium metabisulfate, glyceraldehyde, oxalic acid, ascorbic acid and sodium nitrite were reagent grade from Fisher Scientific. Octanesulfonic acid sodium salt from Eastman and sodium hydroxide and hydrochloric acid from Mallinckrodt were reagent grade.

Compounds studied

The compounds utilized in the study including epinephrine bitartrate, epinephrine-2-sulfonate, norepinephrine bitartrate, dopamine · HCl, isoproterenol bitartrate and bupivacaine · HCl were from Sterling Drug, while 3,4-dihydroxybenzylamine · HBr was from Sigma.

Apparatus

The HPLC–ED system consisted of a Waters M6000 pump at a flow-rate of 1.2 ml/min and a Bioanalytical Systems LC-4 amperometric detector set at +0.65 V with a 50 nA/V sensitivity. This was equipped with a TL-5 thin-layer glassy carbon working electrode *versus* a Ag/AgCl reference electrode. A Micromeritics 725 autoinjector with a 10-μl injection loop and a Fisher Recordall 5000 strip-chart recorder with input set at 1.0 V were used.

Chromatographic conditions

HPLC columns used were 25 cm × 4.6 mm I.D. stainless steel, 5-μm and 10-μm ODS-3 from Whatman. The mobile phase consisted of water–methanol–2 *M* sodium phosphate monobasic–ethylenediaminetetraacetic acid, disodium salt (20 mg/ml in water)–85% phosphoric acid–octanesulfonic acid, sodium salt (900 ml:50 ml:50 ml:2 ml:0.4 ml:0.4 g) with an apparent pH of 3.2.

Epinephrine standard linearity

An epinephrine bitartrate standard solution was accurately prepared at about

0.2 mg/ml in mobile phase and 5.0 ml was diluted to 100.0 ml with mobile phase. This solution was further diluted by transferring 5.0, 7.0, 10.0, 15.0 and 20.0 ml to 50 ml volumetric flasks and filling to volume with mobile phase.

Linearity of recovery from simulated samples

Duplicate samples were prepared at 80, 100 and 120% of the epinephrine bitartrate claim values in placebo and diluted with mobile phase to 0.002 mg/ml for the 100% samples for analysis.

Specificity

Separation of analogues. Solutions of epinephrine bitartrate, epinephrine sulfonate, norepinephrine bitartrate, dihydroxybenzylamine · HBr, dopamine · HCl and isoproterenol bitartrate were prepared in mobile phase at about 0.002 mg/ml and chromatographed individually and combined.

Stressed drug substance. Weighed portions of epinephrine bitartrate were stressed with water, 0.1 *M* hydrochloric acid and 0.05 *M* sodium hydroxide on a steam bath for 4, 4 and 0.5 h, respectively. These solutions were neutralized and diluted for analysis. In addition, a sample was held in water at room temperature for 4 h with a stream of air flowing for comparison to a non-stressed water solution at room temperature.

Stressed placebo

A placebo was prepared containing all constituents except epinephrine bitartrate and held a 70° for 42 h. It was cooled and diluted for analysis while a second portion, fortified with epinephrine bitartrate to give a 100% sample, was also analyzed.

Resolution factor

A resolution factor standard was prepared containing 0.002 mg/ml each of epinephrine bitartrate and dopamine · HCl.

Void volume indicators

Solutions of glyceraldehyde, oxalic acid, ascorbic acid, sodium nitrite and sodium metabisulfite were chromatographed in order to determine the column void volume.

RESULTS AND DICUSSION

Data which are to be submitted to regulatory authorities as obtained from pharmaceutical stability studies and for release of clinical supplies must be supported by appropriate method validation information. This is true whether the assay is performed by titration, spectrophotometry, electrophoresis or chromatography, either thin-layer chromatography (TLC), gas chromatography (GC) or HPLC. It also is true irrespective of the method of detection utilized in the latter cases including amperometric detection for HPLC. In dosage form studies sufficient levels of drug can normally be realized for effective detection by ordinary UV–visible means whereas the added specificity inherent in electrochemical detectors gives an advantage to this detection mode.

TABLE I

LINEARITY OF RECOVERY OF EPINEPHRINE BITARTRATE FROM SIMULATED SAMPLES

Added (mg)	*Found (mg)*	*Recovery (%)*
0.0162	0.0162	100.0
0.0162	0.0165	101.8
0.0203	0.0204	100.5
0.0244	0.0242	99.2
0.0244	0.0246	100.8
S.D.	0.000185 Average	100.6
Correlation coefficient	0.9989	

Linearity of the detector response-concentration relationship for the present method was found. Concentrations ranging from 0.001 to 0.004 mg/ml epinephrine bitartrate, or one-half to two times the nominal standard concentration, gave a correlation coefficient of 0.99986 as related to peak response.

Accuracy of the method is illustrated by results in Table I where placebos were fortified with epinephrine bitartrate at 80, 100 and 120% of the claim value. The average percent recovery of 100.6% as well as the high linear correlation coefficient and low standard deviation of mg found attest to the acceptable method accuracy.

Suitable method precision was also obtained in contrast to the supposed unreliability of amperometric detection methods. These results shown in Table II for four replicate assays on each of two lots of drug product include extremely low percent relative standard deviation (R.S.D.) values on the level of the best UV detection available.

Method specificity was obtained from two lines of evidence: chromatography of chemically stressed drug substance and separation of structural analogs using the proposed method. Recoveries for neutral, acid, base and air stressed epinephrine bitartrate in solution are shown in Table III with no interfering peaks found in any of their respective chromatograms. Fig. 1 shows the baseline separation obtained for a mixture of six catechol analogues studied using the proposed method. These were

TABLE II

REPLICATE ANALYSIS OF TWO COMMERCIAL BATCHES OF BUPIVACAINE · HCl WITH EPINEPHRINE

Determination	*Epinephrine base found (mg/ml)*	
	Batch 1	*Batch 2*
1	0.00544	0.00561
2	0.00550	0.00561
3	0.00556	0.00561
4	0.00550	0.00561
Average	0.00550	0.00561
R.S.D. (%)	0.89	–

TABLE III

EPINEPHRINE BITARTRATE RECOVERY FROM CHEMICALLY STRESSED DRUG SUBSTANCE

Stress conditions	*Time (h)*	*Recovery (%)*
0.1 *M* Hydrochloric acid-steam bath	4	96.6
0.05 *M* Sodium hydroxide-steam bath	0.5	55.6
Water-steam bath	4	98.5
Aeration-room temperature	4	97.8

each separated by a minimum of 4 min, even for such similar compounds as norepinephrine and dihydroxybenzylamine. The compounds clearly elute in order of decreasing polarity with increasing number of methylene or methyl units.

A chromatogram of a bupivacaine with epinephrine bitartrate sample using the conditions described is shown in Fig. 2A while an epinephrine bitartrate standard at 0.002 mg/ml is shown in Fig. 2B. The large peak at the solvent front in the sample chromatogram represents readily oxidizable excipients. The retention characteristics of bupivacaine in the sample were studied by tandem UV detection which showed no elution from the column under the conditions of the method owing to the high aqueous

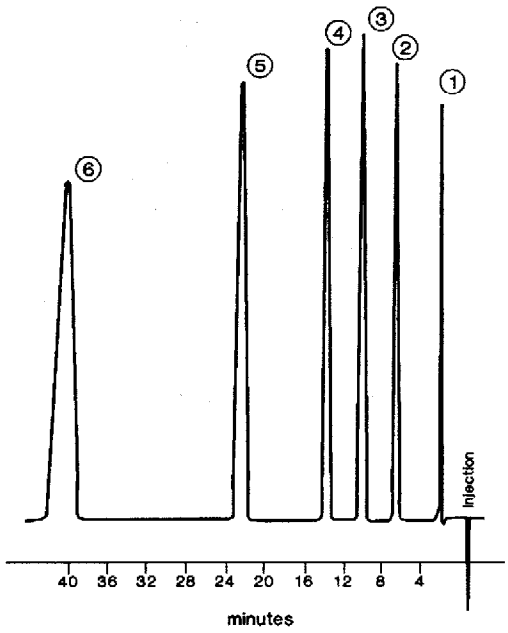

Fig. 1. Chromatogram of catecholamine mixture separated using a mobile phase of: water–methanol–2 *M* sodium phosphate monobasic–ethylenediaminetetraacetic acid, disodium salt (20 mg/ml in water)–85% phosphoric acid–octanesulfonic acid, sodium salt (900 ml:50 ml:50 ml:2 ml:0.4 ml:0.4 g) on a 10-μm ODS-3 column at 1.2 ml/min. These include: epinephrine-2-sulfonate (1), norepinephrine (2), epinephrine (3), 3,4-dihydroxybenzylamine (4), dopamine (5) and isoproterenol (6).

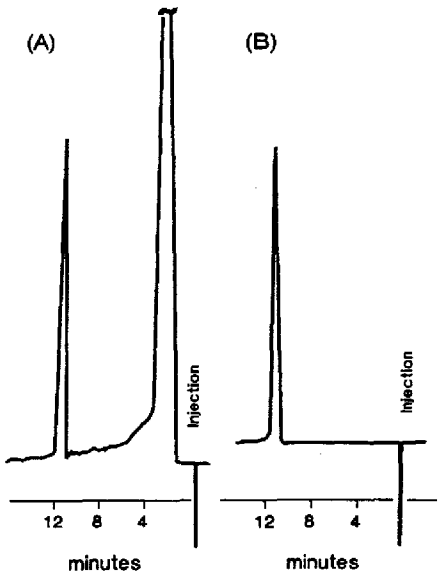

Fig. 2. Chromatograms of a bupivacaine · HCl with epinephrine bitartrate sample (A) and an epinephrine bitartrate standard at 0.002 mg/ml (B) showing the epinephrine retention time of about 10 min. HPLC conditions as described in Fig. 1.

content (95%) and the presence of the pairing ion in the mobile phase. Even if the compound had eluted, it would not have been detected because of the additional selectivity afforded by the amperometric detector. Aliphatic amines do not readily oxidize under these conditions and thus, as opposed to aromatic amines, bupivacaine did not represent a potential interference.

A stressed placebo study using the method described in Fig. 1 gave very interesting results which reinforce the regulatory edict that this type of study be included in submissions. In the case of epinephrine bitartrate and bupivacaine · HCl an unstressed placebo chromatogram is shown in Fig. 3A. When this placebo was stressed at 70° for 42 h the additional peak seen at 5, 8 and 19 min in Fig. 3B were found with amperometric detection. Utilization of a lower water content or lower pairing-ion concentration resulted in interference fromm peaks in the stressed placebo with the main epinephrine peak. This was alleviated using the conditions described as shown in Fig. 3C, an epinephrine-spiked stressed placebo, showing no interference. This exercise serves to emphasize the importance of making these determinations, a conclusion not frequently encountered in the literature.

The question of accurately measuring the void volume was confronted in determining capacity factor (k') values using amperometric detection. Commonly in UV detection systems a concentrated salt solution is used, such as 1 mg/ml sodium nitrate which would show no retention and elute with the solvent. Under conditions of the current method, however, a very polar electrochemically active substance was sought. Among those tested, it was found that a 1 mg/ml solution of sodium metabisulfate gave the best indication of the void volume as shown in Table IV. Sodium nitrite, glyceraldehyde, oxalic acid and ascorbic acid were retained by the column past the void volume although they were detectable.

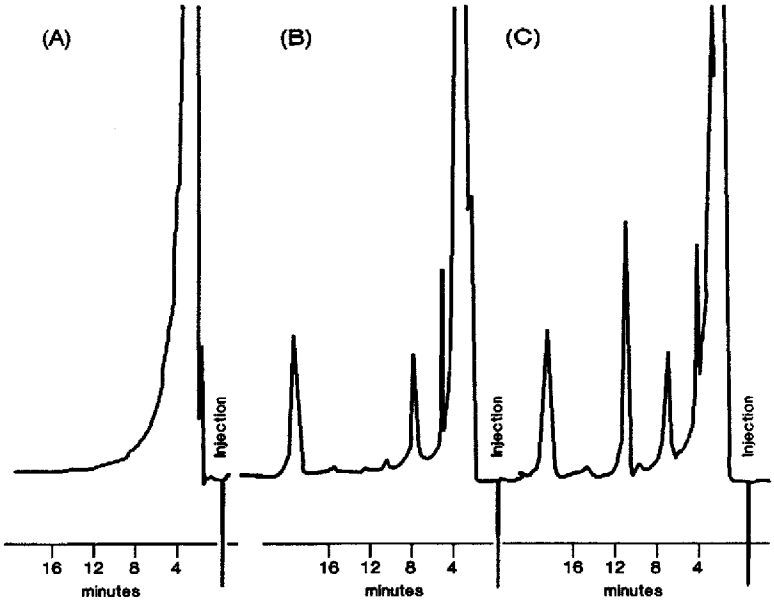

Fig. 3. Chromatograms of unstressed bupivacaine · HCl with epinephrine bitartrate placebo (A), placebo stressed at 70° for 42 h showing degradation peaks at 5, 8 and 19 min (B) and the same stressed placebo with epinephrine bitartrate added showing no interference (C). HPLC conditions as described in Fig. 1.

TABLE IV

VOID VOLUME INDICATORS

Indicator	*Concentration (mg/ml)*	*Result*
Glyceraldehyde	0.001	Eluted after void
Oxalic acid	0.001	Eluted after void
Ascorbic acid	0.001	Eluted after void
Sodium nitrite	10.0	Eluted at void with second peak after void
Sodium metabisulfite ($Na_2S_2O_5$)	1.0	Eluted at void

The amperometric detection method developed for epinephrine in the injectable drug product Marcaine · HCl with epinephrine took advantage of the selectivity of this detector in avoiding the potential bupivacaine peak although it faced the additional problem of detection of stressed placebo products which were not observed with conventional UV detection. The validation results in terms of accuracy, precision, linearity, specificity and ruggedness pass the normal criteria for suitability much the same as any dosage form method would and suggest that amperometric detection should be considered when an added degree of selectivity or sensitivity is desired.

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