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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE RADIOMETRIC DETERMINATION OF [^{14}C]BUCROMARONE IN HUMAN PLASMA UTILIZING NON-RADIOLABELED BUCROMARONE AS AN INTERNAL STANDARD

D.W. EVERETT*, J.E. FOLEY, S.M. SINGHVI and S.H. WEINSTEIN

Squibb Institute for Medical Research, P O Box 4000, Princeton, NJ 08543-4000 (U S A.)

and

S.J. WARRINGTON

Charterhouse Clinical Research Unit Limited, London EC1M 6HR (U K)

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SUMMARY

A novel radiometric high-performance liquid chromatographic (HPLC) method was developed for the determination of [^{14}C]bucromarone in human plasma. The procedure involved the addition of non-radiolabeled bucromarone hydrochloride to each plasma sample as an internal standard; the plasma sample was then extracted, and the bucromarone was separated from its metabolites and endogenous compounds by reversed phase HPLC. The concentration of [^{14}C]bucromarone in each plasma sample was calculated from the ratio of the amount of radioactivity in the eluate fraction corresponding to bucromarone and the peak height of the ultraviolet absorbance (210 nm) of the non-radiolabeled bucromarone used as an internal standard. The lower limit of quantitation for bucromarone free base in this assay was 8 ng/ml when [^{14}C]bucromarone succinate had a specific activity of 0.5 μCi/mg. The coefficients of variation for the experimentally determined concentrations of bucromarone in spiked plasma samples were 6.8 and 14.3% at concentrations of 80 and 20 ng/ml, respectively. This method was used to determine concentrations of bucromarone in the plasma of healthy volunteers who were given intravenous infusions of [^{14}C]bucromarone succinate. In general, the methodology should be applicable to any radiolabeled compound that possesses appreciable ultraviolet absorbance.

INTRODUCTION

A variety of analytical problems are encountered in pharmacokinetic, biopharmaceutic, and drug metabolic studies, some of which can be solved only with the aid of radiolabeled drugs. Radiolabeled compounds in biological samples are com-

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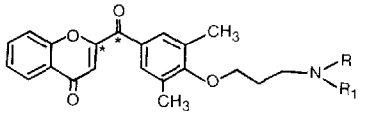
monly quantitated by first developing a method to separate the components in the sample by a suitable chromatographic technique, often high-performance liquid chromatography (HPLC), which results in a radiochromatogram of the drug and its metabolites. From the fraction of the total radioactivity contained within each radioactive peak and the specific activity of the administered drug, the concentration of each component in the original sample can be calculated.

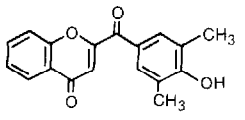
An alternative method for the quantitation of radiolabeled compounds in biological samples combines the technique of inverse isotope dilution analysis with HPLC [1–5]. In that methodology, a certain quantity of the non-radiolabeled compound is added to the biological sample containing the radiolabeled compound. After separation of the components of the sample by HPLC, the concentration of radiolabeled compound in the analytical sample is calculated by use of the principles of isotope dilution analysis.

The use of internal standards in quantitative HPLC analyses to compensate for various analytical errors is widely accepted [6]. The ideal internal standard should be as close as possible to the compound of interest in structure and physical properties. This fact has been recognized in the use of stable isotopes for quantitation by mass spectroscopy [7], but appears to have been overlooked in the quantitation of radiolabeled compounds by HPLC. A search of the literature failed to find any examples of the use of the non-radiolabeled form of a compound as an internal standard for the radiometric HPLC analysis of the same radiolabeled compound.

Bucromarone, designated chemically as 2-[4-[3-(dibutylamino)propoxy]-3′,5′-dimethylbenzoyl]chromone (Fig. 1), is similar in structure to amiodarone, an antiarrhythmic agent. A sensitive and reliable assay for [^{14}C]bucromarone concentrations in human plasma was required for biopharmaceutic studies. These biopharmaceutic studies provided an opportunity to demonstrate the use of a non-radiolabeled compound as an internal standard for the same radiolabeled



Compound	R	R_1
Bucromarone	n-butyl	n-butyl
Monodesbutylbucromarone	H	n-butyl
Didesbutylbucromarone	H	H



Benzopyran-4-one-2-benzoyl-
3′, 5′-dimethyl phenol

Fig. 1. Structures of bucromarone and three of its possible metabolites. The asterisks indicate positions labeled with ^{14}C in radiolabeled bucromarone.

compound. Therefore, an HPLC radiometric assay for [^{14}C]bucromarone was developed, which used non-radiolabeled bucromarone as an internal standard.

EXPERIMENTAL

Materials

Bucromarone hydrochloride, [^{14}C]bucromarone succinate, monodesbutylbucromarone, didesbutylbucromarone, and benzopyran-4-one-2-benzoyl-3′,5′-dimethyl phenol (Fig. 1) were obtained from Transphyto, (Clermont-Ferrand, France). The radiochemical purity of [^{14}C]bucromarone succinate was 93.0%, as determined by HPLC. Reagent-grade 1-pentanesulfonic acid, sodium salt (PSA) was obtained from Eastman Kodak (Rochester, NY, U.S.A.). Glycine was obtained from Sigma (St. Louis, MO, U.S.A.). HPLC-grade acetonitrile and methanol were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Type I reagent-grade water was obtained from a Barnstead Nanopure II® water purification system (Sybron, Boston, MA, U.S.A.). Drug-free plasma from healthy volunteers was obtained from Biological Specialty (Landsdale, PA, U.S.A.). Hydrocount® liquid scintillation cocktail was purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.).

Instrumentation

A Model 1090A HPLC unit (Hewlett-Packard, Avondale, PA, U.S.A.), equipped with an autoinjector and a diode-array UV detector, was used. Chromatography was performed with a 6-μm Zorbax® CN column (25 cm$\times$0.46 cm I.D.; Dupont, Wilmington, DE, U.S.A.), equipped with a Brownlee guard column. The guard column consisted of a cyano guard cartidge (3.0 cm$\times$0.46 cm I.D.) contained within a stainless-steel holder (Rainin Instruments, Woburn, MA, U.S.A.). Peak height of chromatographic peaks was determined by use of a Model 4416 chromatography data system (Nelson Analytical, Cupertino, CA, U.S.A.). Standards were weighed with a Cahn Model 25 electrobalance (Ventron, Cerritos, CA, U.S.A.). For collection of the effluent from the HPLC column, a Model 201B microprocessor-controlled fraction collector (Gilson Medical Electronics, Middleton, WI, U.S.A.) was used. Radioactivity was determined by use of a Tri-Carb® Model 2425B liquid scintillation counter (Packard Instruments, Downers Grove, IL, U.S.A.).

Analysis of plasma samples

A 2.0-ml aliquot of each plasma sample or standard to be analyzed was pipetted into a 25-ml glass centrifuge tube, equipped with a PTFE-lined screw cap. To each plasma sample were added 25 μl of a solution of the internal standard (bucromarone hydrochloride in methanol, 1 mg/ml). The sample was mixed, and 2 ml of 0.1 *M* glycine buffer, pH 9.0, were added (the glycine buffer was prepared by dissolving 7.51 g of glycine and 5.85 g of sodium chloride in 1 l of water and adjusting the pH to 9.0 with 1.0 *M* sodium hydroxide). *n*-Hexane (12 ml) was then added, the tubes were tightly capped and shaken mechanically for 20 min. Following centrifugation for 10 min at 700 *g* (10°C), the tubes were placed in a

mixture of dry ice and acetone to freeze the aqueous layer (lower), and the organic layer (upper) was transferred to a clean glass culture tube; the hexane was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 150 μl of HPLC mobile phase, and 125 μl were injected onto the HPLC column. After the chromatographic separation of bucromarone from its metabolites, the eluate fraction which corresponded to bucromarone was mixed with 15 ml of liquid scintillation cocktail, and the amount of radioactivity in the bucromarone fraction was determined.

Preparation of standards

Control human plasma was spiked with [^{14}C]bucromarone succinate (specific activity 0.5 μCi/mg) to obtain final concentrations ranging from 10 to 1000 ng/ml (see Table I). Extraction of standards was carried out as described in *Analysis of plasma samples.*

Chromatographic conditions

The chromatographic conditions for the HPLC radiometric analysis of [^{14}C]bucromarone are: column, Zorbax CN; mobile phase, acetonitrile–methanol–0.01 *M* aqueous PSA, pH 3.0 (4:3:3, v/v); flow-rate, 1.0 ml/min; wavelength, 210 nm; temperature, ambient; run-time, 15 min; injection volume, 125 μl.

Calculations

A ratio was calculated for each standard by dividing the radioactivity (dpm) contained in the eluate fraction corresponding to bucromarone by the UV detector response (peak height in μV) for bucromarone. This ratio is analogous to the peak-height ratio used in non-radioactive chromatographic methods which employ an internal standard. The peak height was proportional to the amount of non-labeled bucromarone (internal standard) present in the standard; carrier bucromarone was added in large excess compared to the small amounts of [^{14}C]bucromarone present in the concentration range of the assay.

A response factor (RF) for each standard was calculated by dividing the concentration of [^{14}C]bucromarone succinate (ng/ml) in that standard by the ratio described above (see Table I). A mean RF value was calculated from the RF values for all of the standards. This mean RF value was used to calculate the concentration of [^{14}C]bucromarone succinate in the unknown sample as follows:

$$\frac{\text{bucromarone radioactivity (dpm)}}{\text{bucromarone peak height } (\mu\text{V})} \times \text{mean RF}\left(\frac{\text{ng } \mu\text{V}}{\text{ml dpm}}\right)$$

$$= \text{bucromarone succinate concentration (ng/ml)}$$

The concentration of bucromarone free base was then calculated by multiplying the concentration of bucromarone succinate by 0.797.

[^{14}C]Bucromarone succinate administration to volunteers

Six healthy subjects were each given a single 100-mg dose of [^{14}C]bucromarone succinate (specific activity, 0.5 μCi/mg) as an intravenous infusion over a period

of 10 min. A full disclosure of the nature and potential risks of the study was made, and informed consent was obtained from each subject before enrollment.

An electrocardiogram was determined continuously for each subject for the first 4 h after dosing; blood pressure and heart rate were monitored at frequent intervals throughout the study period. Blood samples were collected immediately before and 5, 10, 20, and 40 min and 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 9.0, 12.0, 24.0, 48.0, 72.0, and 96 h after the intravenous infusion of [^{14}C]bucromarone succinate was completed. Plasma was prepared from each blood sample and stored frozen at −20 °C until analyzed by HPLC.

Pharmacokinetic analysis

Pharmacokinetic parameters were calculated from concentrations of bucromarone in plasma determined after an intravenous dose of bucromarone. Least-squares regression and RS/1 software (BBN Software Products, Cambridge, MA, U.S.A.) were used to estimate the terminal half-life ($t_{1/2\beta}$) of bucromarone in plasma. This value was then used to estimate the area under the plasma concentration–time curve ($AUC_{0\to\infty}$) beyond the last time at which concentrations of bucromarone were detectable. The total systemic clearance and the mean residence time of bucromarone were calculated using standard pharmacokinetic equations [8].

RESULTS AND DISCUSSION

Method validation

Fig. 2 shows typical chromatograms resulting from the HPLC analysis of control human plasma with and without added non-radiolabeled bucromarone. No endogenous substances were found in plasma that had the same retention time (about 13 min) as that of bucromarone.

Table I shows the results of assaying control human plasma samples, which

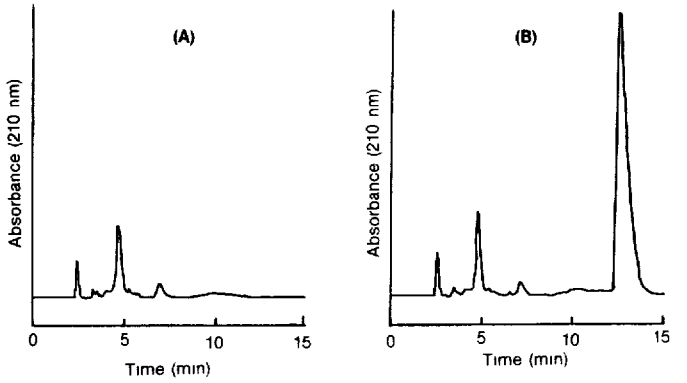


Fig. 2. Typical chromatogram for the HPLC analysis of human plasma with (A) no bucromarone hydrochloride added and (B) 25 μg of non-radiolabeled bucromarone hydrochloride added. The retention time of bucromarone is about 13 min under these conditions.

TABLE I

[^{14}C]BUCROMARONE SUCCINATE STANDARDS IN HUMAN PLASMA

Actual concentration (ng/ml)	Radioactivity recovered (dpm)	Peak height (μV)	Ratio[a] $\times 10^6$ (dpm/μV)	Response factor[b] $\times 10^{-3}$ (ng μV/ml dpm)	Experimentally determined concentration (ng/ml)	Percentage deviation[c]
1000	877	560083	1566	639	940	−6.0
500	490	580244	844	592	506	+1.2
250	216	551850	391	639	235	−6.0
100	85	552861	154	650	92	−8.0
50	51	583265	87	572	52	+4.0
25	21	515628	41	614	25	0.0
10	12	588708	20	491	12	+20.0
				600 ± 55[d]		

[a]Ratio = radioactivity recovered/peak height.
[b]Response factor = actual concentration/ratio.
[c]Percentage deviation = [(experimental concentration − actual concentration)/actual concentration] × 100.
[d]Mean ± S.D.

were spiked with [^{14}C]bucromarone succinate in the concentration range 10–1000 ng/ml (equivalent to 8–800 ng/ml bucromarone free base). The experimentally determined concentrations were in excellent agreement with the actual concentrations. The excellent agreement between response factors calculated at each concentration (coefficient of variation = 9.2%) indicates linearity throughout the concentration range of the assay.

The lower limit of quantitation for a radiometric assay is dependent upon: (1) the specific activity of the analyte (in this case [^{14}C]bucromarone) and (2) the variability in the determination of the background value or noise for the assay. In this study, [^{14}C]bucromarone succinate had a specific activity of 0.5 μCi/mg; therefore, at a concentration of 10 ng/ml (8 ng/ml free base) in plasma, there were 22 net dpm in a 2.0-ml plasma sample. Of this 22 net dpm, about 12 dpm were recovered after extraction and chromatography (Table I).

To determine the variability of the background for this assay, drug-free plasma samples from six subjects were analyzed on separate days. The mean value (± S.D.) of the background for these control plasma samples was 24.7 ± 1.5 cpm. Thus, 95% of human plasma samples which contain no [^{14}C]bucromarone will be within the range of 24.7 ± 3 cpm. A value of 10 cpm above background (about 12 dpm under the conditions of the analysis) would be appreciably greater than the 95% confidence interval for the background value. Therefore, the lower limit of quantitation was conservatively considered to be 8 ng/ml free base.

The recovery of [^{14}C]bucromarone from human plasma was determined by spiking normal human plasma with [^{14}C]bucromarone succinate. After samples were extracted with *n*-hexane, evaporated to dryness, and reconstituted in mobile phase, 73 and 69% of the bucromarone was recovered at concentrations of 80 and 800 ng/ml, respectively.

The specificity of the assay was demonstrated by the ability to distinguish buc-

romarone from three of its possible metabolites (Fig. 3) and the absence of interference by endogenous substances (Fig. 2).

To determine the precision and accuracy of the method in human plasma, five replicate, spiked samples were analyzed at concentrations of 20 and 80 ng/ml bucromarone (Table II). The coefficient of variation (less than 15%) and the accuracy of the method (a deviation of less than 8%) in human plasma were excellent.

The mechanics of the isotope dilution analysis described in the Introduction and the HPLC radiometric assay for [^{14}C]bucromarone described here are similar; both require addition of non-radiolabeled carrier to the sample, sample clean-up, separation of the mixture by HPLC, collection and integration (or determination of peak height) of the carrier peak, and measurement of the radioactivity in this eluate fraction. However, the isotope dilution method requires external standards containing only non-radiolabeled carrier, while the assay described here is a true internal standard assay, requiring a ratio of standards (both non-radiolabeled and radiolabeled compound) and as such should, in general, give a

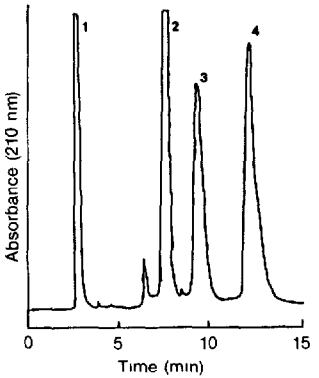


Fig. 3. Separation of bucromarone and three of its possible metabolites in the HPLC system used for the assay of [^{14}C]bucromarone. Peaks: 1 = benzopyran-4-one-2-benzoyl-3′,5′-dimethyl phenol; 2 = didesbutylbucromarone; 3 = monodesbutylbucromarone; 4 = bucromarone.

TABLE II

PRECISION AND ACCURACY OF THE METHOD FOR THE DETERMINATION OF BUCROMARONE IN HUMAN PLASMA

Actual concentration (ng/ml)	Experimentally determined concentration[a] (ng/ml)	Precision (C.V.)[b] (%)	Accuracy (percentage deviation)[c]
80	74 ± 5	6.8	−7.5
20	21 ± 3	14.3	+5.0

[a] Mean ± S.D. (n = 5).

[b] C.V. = coefficient of variation.

[c] [(Experimental concentration − actual concentration)/actual concentration] × 100.

more precise and accurate assay. The isotope dilution method is useful for the quantitation of radiolabeled compounds for which no radiolabeled standard is available; e.g., a metabolite of a radiolabeled drug. Only the non-radiolabeled reference compound is required to quantitate the metabolite.

Both the isotope dilution method and the internal standard method of HPLC analysis require the addition of a relatively large excess of carrier to the sample. The reasons for the addition of a large excess of carrier are: (1) the anomalous chromatographic behavior often encountered at very low concentrations of an analyte is avoided; (2) non-radiolabeled carrier serves as a chromatographic reference for the traces of radiolabeled analyte present; (3) low amounts of components, which are endogenous to plasma, will not interfere in the assay; and (4) to insure that the amount of radiolabeled analyte is too small to interfere in the determination of the peak height of the carrier.

Application to a clinical study

Concentrations of bucromarone in plasma from normal subjects were detectable up to 6 h after an intravenous infusion of 100 mg of [^{14}C]bucromarone succinate; a semilogarithmic plot of mean bucromarone concentrations in plasma versus time after the intravenous dose of [^{14}C]bucromarone succinate is shown in Fig. 4. The average ($\pm$S.E.M.) systemic clearance, terminal half-life, and mean residence time for bucromarone were 6.84 $\pm$ 0.78 l/min, 0.85 $\pm$ 0.06 h and 1.2 $\pm$ 0.1 h, respectively. These data indicate that bucromarone was rapidly cleared from the body after intravenous administration to healthy volunteers.

General applicability of the method

The method described in this report should be generally applicable to the analysis of any radiolabeled drug in biological fluids, provided the drug possesses a

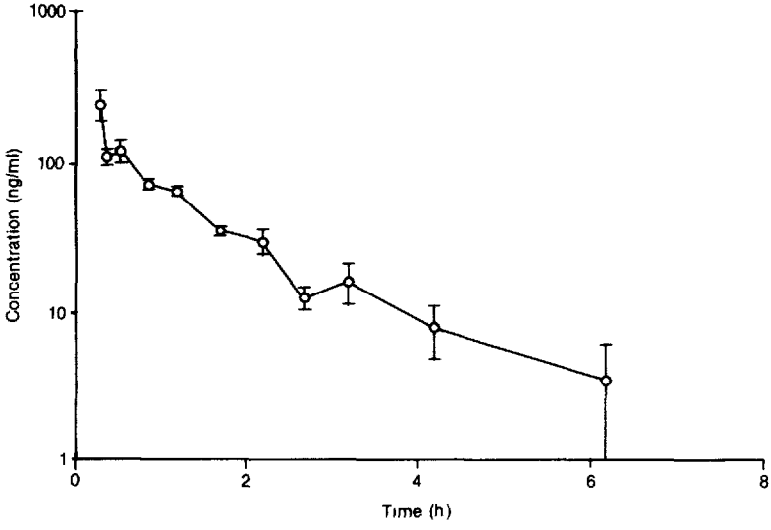


Fig. 4. Concentrations of [^{14}C]bucromarone (as the free base) in human plasma after a single 100-mg dose of [^{14}C]bucromarone succinate given as an intravenous infusion over a period of 10 min. Each value is the mean $\pm$S.E.M. of six subjects.

reasonable ultraviolet absorbance. The sensitivity of the analysis will be determined by the specific activity of the analyte. Small amounts of endogenous components that may elute at the retention time of the analyte will usually not interfere in the assay because of the large amount of internal standard added.

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