Journal of Chromatography, 131 (1977) 357–363

CHROM. 9553

DETERMINATION OF VARIOUS DRUGS IN RODENT DIET MIXTURES

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(Received June 21st, 1976)

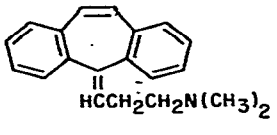
SUMMARY

Methods employing solvent extraction, thin-layer chromatography, gas–liquid chromatography, and UV spectrophotometry are described for the quantitative determination of halofenate, cyclobenzaprine and sulindac in rodent diet mixtures. Halofenate was hydrolyzed to its free acid derivative and converted to a methyl ester prior to assay. The drugs were shown to be stable when stored in food mixtures at room temperature for seven days. Diet mixtures containing the three drugs were demonstrated to be uniformly mixed by the procedure employed.

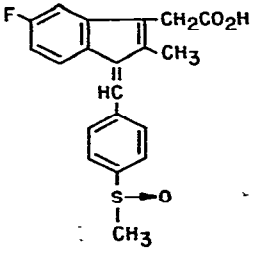
INTRODUCTION

Administration of drugs to laboratory animals by incorporation into the diet is often resorted to in long-term studies to reduce the expense and inconvenience of individual manual dosing. In such cases, the dose of drug will, of course, be proportional to the weight of diet ingested if two conditions are met: (1) the diet–drug mixture must be uniformly mixed and (2) the drug must be stable in the diet mixture under the storage conditions used. Thus it is important to have suitable analytical methods available to demonstrate that these conditions have been fulfilled.

The present report describes methods used to measure three drugs in rodent diet as part of long-term toxicity studies of the compounds. The three drugs chosen for study —cyclobenzaprine, sulindac and halofenate— are basic, acidic and neutral organic compounds, respectively, and thus the assay procedures described may be generally applicable to a wider range of pharmaceuticals in diet mixtures.



CYCLOBENZAPRINE



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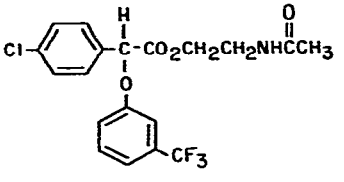

HALOFENATE

MATERIALS AND METHODS

Chemicals and reagents

Halofenate [2-acetamidoethyl(*p*-chlorophenyl)(*m*-trifluoromethylphenoxy)acetate], halofenate free acid [(*p*-chlorophenyl)(*m*-trifluoromethylphenoxy)acetic acid, cyclobenzaprine (N,N-dimethyl-5H-dibenzo[*a,d*]cycloheptene-$\Delta^{5,\gamma}$-propylamine hydrochloride) and sulindac {*cis*-5-fluoro-2-methyl-1-[*p*-(methylsulfuryl)-benzylidenyl]-indene-3-acetic acid} were of high purity.

[5,10,11-$^{14}C_3$]Cyclobenzaprine (sp. act. = 14.1 μCi/mg), [carboxyl-^{14}C]halofenate (sp. act. = 5.3 μCi/mg), [carboxy-^{14}C]halofenate free acid (sp. act. = 6.7 μCi/mg), and [methylene-^{14}C]sulindac (sp. act. = 4.6 μCi/mg) were shown to be radiochemically pure by thin-layer chromatography (TLC) and were used as internal standards for extraction and TLC.

The two-carbon side-chain anlaog of cyclobenzaprine, N,N-dimethyl-5H-dibenzo[*a,d*]cycloheptene-$\Delta^{5,\gamma}$-ethylamine hydrochloride, and the dichloro analog of halofenate free acid, (2,4-dichlorophenyl)(3-trifluoromethylphenoxy)acetic acid, were used as internal standards for gas–liquid chromatography (GLC).

All solvents were analytical-reagent grade and used as purchased. Diazomethane was generated from N-nitroso-N-methylurea (Aldrich, Milwaukee, Wisc., U.S.A.) by addition of 3 *N* KOH. The diazomethane was extracted with diethyl ether and used without further purification.

Gas–liquid chromatography

GLC analyses were performed on a Hewlett-Packard Model 810 gas chromatograph equipped with a hydrogen flame ionization detector. The column was a 6 ft. × 4 mm I.D. glass spiral packed with 1.5% OV-17 on Gas-Chrom Q, 80–100 mesh. The temperatures of the flash heater, column, and detector for halofenate analysis were 210, 190 and 250°, respectively; for cyclobenzaprine analyses, the respective temperatures were 260, 220 and 285°. Flow-rates for the helium (carrier gas), hydrogen and air for both assays were 100, 50 and 450 ml/min, respectively.

Radioassay

Samples were counted with a Packard Model 3310 liquid scintillation spectrometer using a dioxan-based counting medium[1]. The counting efficiency was determined by the channel ratio technique.

Spectrophotometric assay

Spectrometry was performed on a Beckman Type DK-2A recording spectrophotometer.

Thin-layer chromatography

TLC was performed on 250-μm silica gel-coated glass plates with inorganic phosphor (Analtech, Wilmington, Del., U.S.A.). Plates were washed by developing with acetone before use. The composition of the solvent systems employed was as follows: (I) benzene–methanol (6:1); (II) acetone–ammonia (100:1); (III) chloroform–acetic acid (95:5). Radioactivity was located on the plate with a Packard Type 7201

radiochromatogram scanner. Cyclobenzaprine and sulindac were visualized under UV illumination.

Preparation of drug–meal diet mixture. The prepared meal consisted of Purina laboratory chow to which 1 % (w/w) of Wesson oil was added to reduce dust formation during mixing. One kilogram of the prepared meal was weighed. A small portion of the meal was triturated separately in a mortar with the required weighed amount of drug. This was then mixed with the remainder of the meal in a mixer of 5 qt. capacity (Hobart, Troy, Ohio, U.S.A.) for 10 min. This 1-kg premixed sample was then added to the remainder of the meal required for the test (5–16 kg) and the lot mixed for 30 min in a mixer of 110 qt. capacity. After mixing, aliquots of the meal–drug combination were taken from bottom, middle and top locations and the concentration of drug in each was determined.

For stability studies, samples were stored for seven days at room temperature and reanalyzed.

Halofenate assay. A weighed (1.0 g) feed sample was spiked with [^{14}C]halofenate (13,680 dpm/0.1 ml; 1.2 μg) as internal standard. The mixture was shaken with 15 ml heptane–isoamyl alcohol (97:3) for 20 min in a 45-ml glass-stoppered centrifuge tube. After centrifugation, as much of the organic phase as possible was transferred to a tube containing 5 ml of 0.1 *N* NaOH. The tube was shaken gently for 20 min and centrifuged, and the organic phase was discarded. One milliliter of 1 *N* HCl was added to the alkaline phase, and the solution was shaken for 15 min with 10 ml of dichloromethane. After centrifugation, the aqueous (upper) phase was discarded and as much of the organic phase as possible was removed and taken to dryness *in vacuo*. The residue was spotted for TLC in solvent I withthe aid ofa few drops of dichloromethane. After development, the [^{14}C]halofenate spot was scraped off and the drug was eluted by shaking with 5 ml of methanol for 15 min. An aliquot (0.2 ml) of the methanol was counted to determine the recovery at this point in the assay.

After evaporation of 4.0 ml of the remaining methanol, the residue was dissolved in 0.2 ml of ethyl acetate containing 500 μg/ml of the dichloro analog of halofenate used as a GLC internal standard. A few drops of ethereal diazomethane were added and, after keeping the mixture at room temperature for 5 min, excess reagent and solvent were removed with a stream of nitrogen. The residue was dissolved in 0.1 ml of ethyl acetate and a 2-μl aliquot was analyzed by GLC. The retention times for halofenate and the internal standard were 2.5 and 3.4 min, respectively.

A standard curve was constructed from the peak height ratio of halofenate/internal standard *versus* the halofenate concentration obtained by analysis of known amounts of halofenate (100 and 200 μg) added to 1 g of control feed. Peak height ratios were corrected before plotting for the radioactive recovery and the 4.0 ml/5.0 ml aliquot of methanol used. All assays were done in duplicate. Control feed samples were also assayed and gave no interfering peaks.

Halofenate free acid assay. A possible breakdown product of haloferate was its free acid analog. Since the basis for the halofenate assay necessitated its conversion to the free acid by alkaline hydrolysis for GLC analysis, the assay method for stability was modified to permit detection of the free acid in the presence of halofenate.

A weighed (1.0 g) feed sample was spiked with [^{14}C]halofenate as before and also with its ^{14}C-labeled free acid (6,587 dpm). The sample was extracted as previously with heptane. As much as possible of the heptane extract was transferred to a tube

containing 5 ml of 0.2 *M* phosphate buffer, pH 8.0. After shaking for 10 min and centrifuging, as much as possible of the organic phase was transferred to a tube containing 5 ml of 0.1 *N* NaOH. The alkaline phase was then assayed for halofenate as before.

The buffer extract, which contained only any free acid present was acidified (1 ml of 1 *N* HCl) and shaken for 10 min with 10 ml of dichloromethane. After separating, the dichloromethane extract was evaporated and subjected to TLC as previously described. At this point, the assay is the same as that used for intact halofenate. All assays were done in duplicate.

Cyclobenzaprine assay. A weighed (1.0 g) feed sample was spiked with [^{14}C]-cyclobenzaprine (30,903 dpm/0.1 ml; 1.0 μg) as internal standard in a 50-ml glass-stoppered centrifuge tube. After addition of 5 ml of 0.5 *N* NaOH and 25 ml of heptane–isoamyl alcohol (97:3), the tube was shaken for 30 min and centrifuged. As much of the organic phase as possible was transferred to a tube containing 5 ml of 0.1 *N* HCl, which was shaken for 10 min and centrifuged. The organic layer was carefully aspirated, and 1.0 ml of 1 *N* NaOH and 10 ml of dichloromethane were added. The tube was shaken for 15 min, centrifuged, the upper aqueous phase aspirated and the dichloromethane transferred to an evaporating tube. After evaporating to dryness *in vacuo*, the residue was redissolved in a small amount of dichloromethane and spotted for TLC in system II. The quenched area corresponding to cyclobenzaprine (R_F 0.60) was scraped off and eluted with 5 ml methanol.

An aliquot (0.2 ml) of the methanol was counted. Four milliliters were evaporated to dryness. The residue was dissolved in 0.1 ml ethyl acetate (containing 250 μg/ml of the internal standard) and 5 μl were used for GLC analysis.

The retention times of cyclobenzaprine and internal standard were 2.1 and 2.8 min, respectively.

The cyclobenzaprine concentration in the feed sample was obtained by reference of the experimental peak height ratio of cyclobenzaprine to internal standard to a standard curve constructed by plotting peak height ratios *versus* cyclobenzaprine concentration obtained by analysis of known amounts of cyclobenzaprine added to 1 g feed samples.

The concentration obtained was corrected for recovery by a factor obtained for each sample from the radioactivity assay. All samples were assayed in duplicate. Blank feed assays showed no peak which interfered in the assay and hence no correction was necessary.

Sulindac assay. A weighed (1.0 g) feed sample, [^{14}C]sulindac (13,050 dpm/0.1 ml; 1.26 μg/0.1 ml) and 10 ml of 0.1 *N* NaOH were shaken for 20 min in a 45-ml glass-stoppered centrifuge tube. Approximately 8 ml of the aqueous phase was transferred (after centrifugation) to a second tube containing 0.9 ml of 1 *N* HCl. *n*-Butyl chloride (15 ml) was added and the tube shaken for 25 min. After centrifuging, as much as possible of the organic phase was transferred to another tube. The extraction was repeated with 10 ml of fresh butyl chloride and as much as possible pooled with the first extract.

The butyl chloride extracts were shaken gently (15 min) with 0.1 *N* NaOH (5 ml). After aspirating the organic phase, the aqueous layer was acidified (1 ml of 1 *N* HCl) and shaken with dichloromethane (10 min). The tube was centrifuged, the aqueous layer aspirated and the organic phase transferred to an evaporating tube. Solvent was removed *in vacuo*.

The residue was dissolved in a small volume of acetone and streaked on a silica gel plate for TLC in solvent system III. The band corresponding to sulindac (R_F 0.30) was located (UV light), scraped off, and eluted with 3 ml of 0.1 *N* NaOH. After shaking and centrifuging, the absorbance at 284 nm was measured.

The concentration of sulindac in the final solution was determined by reference to standards. Concentration in the unknown was then obtained by correcting the drug concentration in the final solution for overall recovery, obtained by counting the eluates from the TLC plates. Readings were also corrected by subtraction of a blank reading (equivalent to about 16 μg/g) obtained on analysis of control feed samples. All assays were in duplicate.

RESULTS AND DISCUSSION

Results of the determination of the overall accuracy and uniformity of mixing of the various drugs in kilogram lots of animal feed are shown in Tables I–III. The mean per cent concentrations of halofenate, cyclobenzaprine and sulindac found were 100.9% ± 2.3, 102.2% ± 7.4 and 101.0% ± 8.3 (mean ± S.D. for eight to twelve different concentrations of each drug), respectively, of the stated concentration values in feed. The coefficients of determination (obtained by squaring the correlation coefficient, R^2) based on the regression of the average concentration found on the average stated concentration were 100%, 99.6% and 99.7%, respectively, for halofenate, cyclobenzaprine and sulindac (data presented in Tables I, II and III), and their respective regression slopes were 1.0, 1.0 and 1.1. These results demonstrate a high degree of accuracy for the assays considered. The mean relative standard deviation (R.S.D.) for all samples of the three drugs taken from different locations of the feed mix was ±2.6% for halofenate, ±6.3% for cyclobenzaprine and ±3.9% for sulindac. These results demonstrate that the samples were uniformly mixed (assuming an acceptable upper limit of ±10%).

Studies on the stability of the three drugs on storage in feed mixtures for seven days at room temperature were also performed. A halofenate feed sample contained 185 mg/kg after storage compared to 203 mg/kg before storage, a decrease of only

TABLE I

ANALYSIS OF HALOGENATE IN RODENT FEED

Stated concentration (mg/kg)	*Concentration found (mg/kg)* *	*Recovery (%)*	*R.S.D. (%)*
88	92.5 ± 3.1	104	3.4
99	103.4 ± 1.3	104	1.3
189	192.9 ± 3.2	102	1.6
206	202.7 ± 3.4	98	1.7
242	240.9 ± 10.4	100	4.3
309	307.4 ± 8.6	99	2.8
515	511.9 ± 10.7	99	2.0
611	617.0 ± 10.6	101	1.7

* Figures represent the mean ± S.D. for six determinations on three samples of each concentration taken from the top, middle and bottom levels of the bulk diet.

TABLE II

ANALYSIS OF CYCLOBENZAPRINE IN RODENT FEED

Stated concentration (mg/kg)	*Concentration found (mg/kg)* *	*Recovery (%)*	*R.S.D. (%)*
18.0	20.5 ± 0.7	114	3.4
18.3	19.8 ± 1.5	108	7.6
44.2	42.2 ± 2.1	95	5.0
46.0	49.2 ± 2.6	107	5.3
48.9	49.0 ± 3.8	100	7.8
58.4	50.1 ± 3.0	86	6.0
87.6	93.5 ± 2.8	107	3.0
98.3	100.5 ± 3.0	102	3.0
115.9	119.1 ± 8.7	103	7.3
152.2	144.3 ± 8.0	95	5.5
222.1	228.3 ± 9.7	103	4.2
301.8	318.8 ± 14.3	106	4.5

* Figures represent the mean ± S.D. for six determinations on three samples of each concentration taken from the top, middle and bottom levels of the bulk diet.

TABLE III

ANALYSIS OF SULINDAC IN RODENT FEED

Stated concentration (mg/kg)	*Concentration found (mg/kg)* *	*Recovery (%)*	*R.S.D. (%)*
47	56.0 ± 2.0	119	3.6
55	50.9 ± 4.3	92	8.4
89	95.5 ± 2.5	107	2.6
106	96.1 ± 4.1	91	4.3
110	101.7 ± 4.8	92	4.7
140	132.5 ± 7.0	95	5.2
186	180.4 ± 7.6	97	4.2
192	190.4 ± 5.3	99	2.8
220	221.4 ± 5.5	101	2.5
285	306.1 ± 6.9	107	2.2
455	476.0 ± 19.6	105	4.1
555	594.2 ± 11.4	107	1.9

* Figures represent the mean ± S.D. for six determinations on three samples of each concentration taken from the top, middle and bottom locations of the bulk diet.

9%. This small reduction in halofenate concentration was apparently a result of hydrolysis since 9 mg/kg of the free acid derivative (equivalent to 11 mg/kg of halofenate) was found in the stored sample. The concentrations of cyclobenzaprine and sulindac did not change appreciably during storage of the feed samples since the per cent difference was only 1.8% and 3.9%, respectively.

The results also demonstrate that the methods presented here for cyclobenzaprine, sulindac and halofenate —three drugs which are basic, acidic and neutral compounds, respectively— are accurate and precise. The standard curves were linear and passed through the origin. The clean-up procedures are more complex than those

required for analysis of these drugs in plasma or urine[1–4] but are necessitated by the presence of higher amounts of extractable endogenous materials in rodent feed which interfere in subsequent analysis. Sources of error inevitable in more complex assays are minimized in these cases by the use of both radioactive and non-radioactive internal standards.

The general principles of the methods described, which depend on solvent partitioning, TLC and GLC or spectrophotometry, may be of value for the analysis of other pharmaceuticals or chemicals in diet mixtures.

ACKNOWLEDGEMENT

The authors wish to thank Mr. John F. Buck for providing the samples of the bulk diet mixtures and Dr. N. R. Bohidar for advice on the statistical treatment of the data.

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